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Maize as production and delivery vehicle of edible vaccines against the enterotoxigenic *Escherichia coli* and the swine transmissible gastroenteritis (TGE)

by

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For Major Program

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Dedicated to my boys

Abstract

Plants are becoming increasingly important as a production system for biopharmaceuticals and industrially important proteins. Early work focused on the production of these novel proteins in crop plants such as tobacco, potato and tomato. The work presented in this dissertation showed that maize can be used as a source and delivery vehicle for edible vaccines. Antigenic proteins from two economically important pathogens, enterotoxigenic strains of *Escherichia coli* (*E. coli*) and the swine transmissible gastroenteritis virus (TGEV) were expressed in transgenic maize.

This study showed that the subunits of the *E. coli* heat labile enterotoxin (LT) can be synthesized in transgenic maize tissues. The B subunits of the labile toxin (LT-B) were expressed in transgenic maize kernels, and data obtained showed that the protein was accumulated, correctly processed and assembled in maize tissue. The role of plant regulatory sequences such as promoters, targeting and retention signals in accumulation of LT-B in transgenic maize kernels was studied. It was established that the seed specific 27 kDa gamma zein promoter achieved a significantly higher level of LT-B expression in kernels compared to the constitutive CaMV 35S promoter. The use of the endoplasmic reticulum retention motif SEKDEL significantly enhanced kernel accumulation of LT-B. The LT-B gene was normally transmitted over three generations as expected. Analysis of the greenhouse and field grown LT-B expressing maize indicated that the level of LT-B expression increased in each successive generation.

Sub-cellular localization of LT-B using immunogold labeling and electron microscopy techniques indicated that the LT-B was encapsulated in transgenic maize kernel

starch. Targeting of antigens to kernel starch granules could have important implications for the expression of other vaccines and industrially important proteins in starch. Maize generated of LT-B had biochemical and biophysical, and immunogenic properties of the bacterial protein. Oral administration of transgenic maize expressing LT-B in BALB/c mice induced elevated titers of serum and mucosal antibodies, which protected the immunized animals from subsequent challenge with LT and Cholera toxin (CT). Transgenic maize induced a significantly higher level of antibody titers compared to an equivalent amount of bacterial LT-B added to non-transgenic maize meal pellets.

Using two synthetic genes for the LT toxin subunits, LT-A and LT-B, a non-toxic derivative of the heat labile toxin, LTK63, was expressed in transgenic maize callus. This mutant toxin assembled in maize callus tissue, showing that complex folding of foreign antigens could be achieved in transgenic maize tissues. This mutant derivative was shown to be more immunogenic than the bacteria derived LT-B.

We fused an N-terminal domain of the spike (S) protein of the swine transmissible gastroenteritis virus to the A subunit of LT, and coexpressed this fusion with LT-B in transgenic maize callus. Expression of the fusion proteins and LT-B was observed in callus.

This work demonstrates that maize, a key ingredient in food and feed industry, can be used as a source and delivery vehicle of functional antigens for use as oral vaccines. Maize holds great potential for the generation of human and livestock vaccines, and this work lays the foundation for the development of vaccines against other pathogens in transgenic maize.

TABLE OF CONTENTS

CHAPTER 1

Introduction

1.1 General introduction

Genetic transformation has added plants to the list of biological systems in which novel proteins can be generated, extending the use of plants beyond the traditional uses of feed and fiber. Plants can be engineered to produce antigens, antibodies, and other proteins for industrial, pharmaceutical and veterinary use. The concept of production of oral vaccines in plants was put forward by Mason et al (1992), who expressed a hepatitis-B surface antigen in transgenic tobacco plants. The surface antigen assembled to give virus-like particles in transgenic tobacco tissue, and had physical properties similar to the human serum derived antigen. Since then, progress has been made in the production of different proteins in plants for use as vaccines to be administered orally or by other routes. Most of these novel proteins were produced in tobacco, potato, tomato and *Arabidopsis*, plants that are easily amenable to *Agrobacterium tumefaciens* based transformation (Mason et al, 1992, 1996 and 1998; Haq et al, 1995; Mcgarvey et al, 1995). There are several benefits in producing antigens for use as edible vaccines in maize. Maize is a major crop world wide, and maize kernel can be used directly as feed and food. This dissertation investigates the possibility of adding maize, an important commercial and staple cereal crop, to the list of plants that are suitable for antigen production.

There are important genetic considerations in the production of a foreign protein in plants. These include regulation and stability of foreign gene expression and the

functionality of the plant synthesized proteins. The research presented in this dissertation investigated the feasibility, from a plant genetics perspective, of using maize as a source of functional antigens. Proteins from microbial and viral pathogens were some of the first examples chosen to show the feasibility of transgenic plant expression systems for pharmaceutical production (Daniell et al, 2001). The rationale was that key immunogenic proteins of major pathogens could be synthesized in plant tissues, and then fed as edible subunit vaccines to humans or livestock.

In this study, antigens from two important pathogens, enterotoxigenic *Escherichia coli* (*E. coli*) and the swine transmissible gastroenteritis virus (TGEV) were used as models. These are gut pathogens for which mucosal immunity is important for protection, which makes them appropriate candidates against which an edible vaccine could be produced. The A and B subunits of the heat labile toxin (LT) from *E. coli*, and the spike (S) protein of TGEV were selected as antigens for expression in maize. The physical and biochemical properties of these proteins allow detection and quantification of gene expression using relatively simple techniques such as enzyme linked immunosorbent assay (ELISA). LT and its B subunit (LT-B) are potent oral immunogens, eliciting high titers of mucosal and serum antibodies upon oral administration. As such, the immunogenicity of maize-synthesized antigens could be easily detected in experimental animals.

In this study, synthetic genes encoding the LT toxin subunits were expressed in transgenic maize. Codon usage in these genes was adjusted for expression in maize. The LT-B gene regulated by the CaMV 35S promoter was transformed into maize callus. Evaluation of callus protein indicated that LT-B was accumulating in transgenic callus tissues and that it was tolerated in maize. The LT-B gene was also fused to the seed-specific

27 kDa maize gamma zein promoter and expressed LT-B in transgenic maize kernels. The level and stability of LT-B gene expression in maize kernels over three generations was determined. The role of promoters in LT-B gene expression in maize was studied by comparing the expression levels achieved by the maize 27 kDa gamma zein and the constitutive CaMV 35S promoters. The role of targeting and retention signals in LT-B accumulation in maize kernels was examined. The integrity of the LT-B produced in maize was determined by testing for biochemical and biophysical properties displayed by the native bacterial protein. The subcellular localization of LT-B in the cells of transgenic maize kernels was established. The immunogenicity of maize derived LT-B was established by oral immunization of laboratory mice. The genes encoding the A and B subunits of LT and non-optimized TGE S protein genes were expressed in transgenic maize callus. Computer-based analysis of the evolutionary relationships of coronaviruses based on the S protein sequences was also made. Regulation of release of genetically engineered organisms into the environment for field-testing is a critical aspect that was addressed in this study.

1.2 Dissertation Organization

This dissertation begins with a broad review of the current literature on production of oral vaccines in plants, and proceeds to research contributions made to this body of knowledge by the present study. The literature review primarily describes the research reported about the production of oral vaccines in plants and highlights the advantages and opportunities presented by the development of this technology. A portion of the review evaluates the characteristics of LT that make it an ideal choice of protein to test in transgenic maize. Justification is also presented on why maize was selected as the crop of choice in this

study. The literature review in this introductory chapter is by no means exhaustive, as each of the subsequent chapters is presented with a literature review in the introduction and discussion.

Chapter 2 presents the expression of LT-B in maize, and examines the role of promoters and ER retention sequences in LT-B expression. The stability of LT-B gene expression over three generations was also studied. This work has been submitted to ***"Molecular Breeding"*** for publication and is currently under review. Chapter 3 presents the role of regulatory sequences, in particular the LT-B and maize zein signal peptides, in subcellular localization of LT-B in transgenic maize kernels. A manuscript describing this work is in progress. The functionality of maize synthesized-LT-B as an immunogen is presented in Chapter 4. This work is currently in press in the journal ***"Transgenic Research"***. Chapter 5 presents progress made towards the development of a subunit vaccine against the swine transmissible gastroenteritis coronavirus. A manuscript summarizing these results is in progress. Chapter 6 is a paper on the evolutionary relationships of coronaviruses, of which TGEV, a subject of my dissertation, is an economically important member. This work was a class assignment (Genet592; Evolutionary Genetics) and recommended for publication in a virology journal by the Instructor Dr Gavin Naylor. This paper examines conservation of antigenic epitopes that were used for the development of a subunit vaccine against TGEV in this study. Chapter 7 contains general conclusions on the work described in this dissertation. Appendix I focuses on the regulatory procedures for the release of transgenic maize containing a biopharmaceutical into the environment.

Chapters 2 through 6 are presented in manuscript format and several names appear in the authorship. Dr Kan Wang is my Major Professor and the principal investigator on this

research project. She provided the guidance and direction for the course of this project. Collaborators on this project include Drs Hugh Mason, Ronald Wesley, Joan Cunnick and Paul Scott. Dr Mason is from the Boyce Thompson Institute of Plant Research at Cornell University and he provided the synthetic LT-B and LTK63 genes used in this study, as well as technical input on analyses of expression of these genes. Dr Ronald Wesley of the National Animal Disease Center (NADC) in Ames, Iowa, provided the porcine respiratory coronavirus (PRCV) total viral RNA from which viral antigens were amplified. He also provided technical input, especially on viral neutralization assays. Dr Cunnick of the Department of Microbiology and Immunology provided hands-on training on animal handling procedures and guidance in experimental design and execution of the mice feeding studies. Dr Paul Scott, a USDA collaborator in the Agronomy Department provided training and technical input on protein analysis and aspects of maize kernel biochemistry. Bronwyn Frame and Sue Ellen Peg of the Plant Transformation Facility performed maize transformation and greenhouse care for all constructs described in this dissertation. Jennifer McMurray was an undergraduate assistant who worked with me on routine analyses presented in the dissertation. Diane Hathaway was a graduate student who worked with me on the edible vaccines project and contributed on the formulation of maize pellets for mice feeding experiments used in Chapter 4. Huixia Shou is a graduate student who contributed with statistical and molecular analyses described in Chapter 2.

1.3 Literature Review

1.3.1 Production of recombinant proteins in plants

Genes can be expressed in many different systems, and the choice of which system to use in the generation of a recombinant protein will depend on the system that offers the most advantages. An ideal system would be the one that produces the safest biologically active protein at the lowest cost (Daniell et al, 2001). There are many potential advantages in the production of antigens for use as vaccines as well as other proteins in plants. From a biological perspective, the plant's eukaryotic protein production system is capable of performing the post-translational modifications such as glycosylation, which, in some cases are essential for biological activity of some proteins. Plants are perceived as more economical than the industrial facilities using fermentation or bioreactor systems (Pen et al, 1996). The amounts of protein produced by plants are comparable to those produced by industrial approaches (Hood et al, 1997). Technology already exists for harvesting and processing plants and plant products on a large scale. The purification requirement can be eliminated from the processing in cases where the recombinant protein is produced in plant tissue that can be used as food or feed (edible vaccine). The proteins can be targeted into intracellular compartments in which they are more stable. Alternatively, they can be expressed directly in those compartments or organelles such as chloroplasts. Production of recombinant proteins in plants minimizes the health risks associated with contamination of the recombinant product with potential human or animal pathogens (Streatfield, 2001).

There are practical, ethical and genetic issues to be considered in the production of these foreign proteins in plants. These aspects include selection of appropriate plant tissues

in which to express and deliver the desired amount of antigenic protein. Novel protein can be produced in green tissue, tubers or grain, depending on the plant. Recombinant protein production has been reported in green tissues of tobacco, alfalfa and soybean. Attractive attributes of these plants include production of large volumes of green tissue in tobacco and alfalfa, which can produce several crops per year by cutting foliage. Annual foliage yields can be 25 and >100 metric tons (MT) per hectare for alfalfa and tobacco, respectively (Daniell et al, 2001). However, these plants have their drawbacks, especially for the purpose of convenient oral vaccine production and delivery. Green leaf tissues in which proteins are produced in these plants tend to have high amounts of phenolic and other potentially toxic compounds. Tobacco and *Arabidopsis* are not palatable, necessitating a purification or extraction step, which may add to the cost of production. An advantage of producing pharmaceuticals in potato is that the tuber is a good tissue for production and storage of proteins. Nevertheless, potato may require cooking to improve its palatability. Arakawa et al (1998) have shown that boiling CT-B generating transgenic potato denatures up to 50% of the antigen.

Grain yields of cereal grains such as wheat, rice and maize are less abundant (3, 6 and 12 metric tons per hectare respectively) than that of the green tissues of tobacco and alfalfa (Daniell et al, 2001). However, high seed production in grains makes scaling up production quite easy. Unlike proteins synthesized in vegetative plant tissues, seed storage proteins are compartmentalized in protein bodies in mature seed. These provide a stable environment devoid of significant amounts of enzyme activity prior to germination. Production of recombinant proteins has the added advantage of long-term storability of functional protein in grain. Levels of scFV antibody in rice seeds or potato tubers did not show a significant

decline after storage at room temperature for 6 months (Daniell et al, 2001).

Maize is a major food and feed ingredient worldwide. Production of an efficacious maize-based oral vaccine will provide effective and inexpensive control of some important gut pathogens, for which mucosal immunity is important for protection. The production of oral vaccines in maize grain or silage presents an opportunity to add value to unprocessed maize and to increase income for farmers. Successful vaccine production in maize will pave the way for the production of other edible vaccines in the crop, contributing significantly to the development of maize as a novel bio-manufacturing system.

1.3.2 Plant genetics considerations

Practical production of a protein in any given system has to be efficient, reliable and stable. The quantity of plant tissue constituting a vaccine dose must be of a practical size for consumption; therefore, transgenic lines should therefore have high expression levels of the gene of interest in appropriate tissues. In addition, the recombinant proteins produced in plants should be structurally and functionally active. Moreover, transgenic plants should be fertile and able to express the transgene predictably over generations. For this reason, expression of a foreign protein in plants requires optimization. Parameters that can be manipulated to achieve high expression levels are discussed below.

1.3.2.1 Stable versus transient expression

Expression of novel proteins in plants can be achieved by either transient expression using modified plant viruses (Mason and Arntzen, 1995) or by stable transformation of the nuclear genome. In transient expression, DNA encoding the gene of interest is introduced into the plant cell where it is recognized by the transcription machinery and expressed. Stable transformation, on the other hand, involves the introduction into the plant cell of a

foreign gene and its stable integration into the genome of the recipient plant. Each of these methods has its advantages and disadvantages. Viral vectors are usually more appropriate for expressing products in leaf tissue for extraction and purification. The expression of foreign protein occurs 2-4 weeks post inoculation, production of antigen is rapid and so is the scale up process (Cramer et al, 1999). Transient expression usually achieves substantially higher levels of protein, but requires inoculation of large number of plants with the recombinant virus. With viral vectors, the inserted gene is often lost over time, which is important for containment of the transgene.

Stable transformation is particularly suitable for expression in edible plant tissue like fruits and tubers, which are not suitable for viral inoculation. Promoters are available that are suitable for organ specific expression and can be developmentally regulated. The ability to sexually cross transgenic lines adds the flexibility of being able to express multiple foreign proteins in the same plant. However, stable transformation requires meticulous selection and breeding of the high expressing transgenic lines, and there are concerns about transgene transfer into relatives. Stable transformation can be achieved, depending on the crop, either by *Agrobacterium tumefaciens*-mediated transformation or microprojectile bombardment, among other methods. For both transformation methods, there is need to construct the expression vector, in which the gene of interest is inserted along with DNA regulatory elements that control mRNA transcription and maturation in plant cells (Tacket and Mason, 1999). For this purpose, promoters and other leader and polyadenylation signals, targeting and retention signals are important. The cassettes may or may not contain a selectable marker gene, to allow for selection of transgenic cells. In both stable and transient transformation methods, the site of transgene insertion is variable, and so is the gene copy

number, although the microprojectile method is associated with a higher number of transgene copies. This results in variability in the level of gene expression in the different transformants. Because insertion sites are random, transgenes may sometimes interfere with endogenous genes, leading to abnormalities in the transgenic plant. For these reasons, selection has to be carried out to determine high expressing non-defective lines.

Stable transformation offers multigenerational stable expression, compared to the use of viral vectors. Expression of recombinant proteins has been achieved by the use of strong promoters, a range of leader and polyadenylation signals and optimization of codon usage for plant expression (Mason et al, 1998; Hood et al, 2001). Some of these regulatory sequences are discussed below.

1.3.2.2 Gene regulatory sequences

1.3.2.2.1 Promoters

Promoters are available for tissue or organ-specific, temporal, and/or induced expression. Promoter elements can be either constitutive or tissue specific, allowing developmental control of expression of the novel protein. Some of the promoters used for foreign gene expression in maize include the maize ubiquitin promoter (Christensen et al, 1992), the Cauliflower Mosaic Virus 35S (CaMV 35S) promoter (Nagy et al, 1985) in its tandem arrangement and the maize seed specific 27 kDa gamma zein promoter (Marks et al, 1985). Seed-specific promoters derived from genes that encode proteins that are abundant in the seed could theoretically result in higher expression of a foreign protein; however, other groups have reported higher expression using constitutive promoters. Hood et al (1997) achieved high avidin expression using the constitutive maize ubiquitin promoter. Expression of a foreign protein with a strong constitutive promoter can have a detrimental effect on cell

processes and plant growth. Mason and co-workers (1998) noticed stunting of transgenic potato plants expressing a high level of the *Escherichia coli* heat labile-B subunit under the control of the enhanced CaMV 35S promoter.

1.3.2.2.2 Subcellular localization

Sub-cellular localization is important for biological activity of a protein. Proper folding and stability of the plant-synthesized protein is critical and is dependent upon the cellular environment where the protein is expressed and stored. For this reason, subunit vaccine components have been targeted to the cell surface, mitochondria, chloroplasts, vacuoles, the endoplasmic reticulum (ER) and the Golgi apparatus. Signal peptides have been used to influence level of transgene accumulation and spatial distribution within transgenic plant tissue. Hood et al (1997), and Zhong et al (1999) have used the barley α-amylase signal peptide to target avidin and aprotinin respectively, to the endoplasmic reticulum during translation (Stachelin and Moore, 1995). In plants, the default pathway for proteins transported through the ER is secretion, hence the proteins were localized in the extracellular space. The expectation was that higher levels of the foreign protein would be obtained if the newly synthesized protein was targeted to the extracellular compartment. Conrad and Fiedler (1998) observed that targeting recombinant antibodies to the secretory pathway significantly increased antibody yield compared to targeting to the cytosol. Targeting avidin to the cytosol was completely toxic to engineered maize cells (Hood et al, 1997). However, a protein's ultimate sub-cellular localization may depend on features other than just the signal peptides, including topological information in the protein itself.

High expression of a gene product, which accumulates in the cytoplasm may result in toxicity to the plant cell (Mason et al, 1998). For this reason, sequestering the protein into a

compartment or organelle or transporting it to the extracellular matrix may allow higher expression levels. While targeting proteins to the intercellular space beneath the cell wall has been shown to increase expression levels, retention in the endoplasmic reticulum can also result in 10- to100-fold higher yields (Conrad and Fiedler 1998). The SEKDEL (Ser-Glu-Lys-Asp-Glu-Leu) or KDEL (Lys-Asp-Glu-Leu) amino acid motifs have been used to retain the foreign proteins in the endoplasmic reticulum. The SEKDEL amino acid motif binds to the SEKDEL receptor in the endoplasmic reticulum (Munro and Pelham, 1987). Haq et al (1995) showed that the SEKDEL motif resulted in significantly higher LT-B accumulation when compared with the LT-B gene without SEKDEL. These authors proposed that the cellular compartmentation of the SEKDEL protein could have facilitated oligomerization of LT-B monomers into pentamers detectable by ganglioside-dependent ELISA.

1.3.2.2.3 Codon usage

The pattern of codon usage in plants is different from that of microorganisms. Altering the composition of the heterologous coding sequence to meet the plant pattern can increase the rate of translation (Kusnadi et al, 1997). The codon bias of a transgene is important in enhanced expression of a foreign protein (Gribskov et al, 1984). Mason et al (1998) synthesized the LT-B gene used in this study with a codon bias optimized for expression in potato and maize and demonstrated that the synthetic gene could be expressed in potato. This group observed that the codon optimized LT-B gene achieved higher LT-B expression in transgenic potatoes than the non-optimized gene.

1.3.2.3 Stability of gene expression and transmission of the transgene

Differences in gene expression between transgenic events have been reported. This

phenomenon is often attributed to differences in gene copy numbers of the transgene and the relative position of insertion in the nuclear genome of the transgenic plants (Matzke and Matzke, 1995). Hood et al (1997) observed variation of avidin expression ranging from 2.1% to 5.7% of aqueous-extractable protein between plants within the same transgenic event. They reported increased gene expression in some transgenic maize plant lines and total silencing of the transgene in others. For this reason, only those transgenic lines with the desired level of expression were pursued. Other workers have reported variation in gene expression between different generations of plants in the same transgenic events. Zhong et al (1999) and Streatfield et al (2001) reported increase in the expression of aprotinin and LT-B respectively between different generations of the same transgenic lines.

A reliable plant antigen production system requires the normal transmission of the foreign gene from generation to generation. Abnormalities in foreign gene transmission have been reported. In two extreme cases, Hood et al (1997) observed that transgenic plants expressing avidin were male sterile as a result of the effect of the transgene, while Sangtong et al (2001) observed that transgenic maize plants expressing the wheat glutenin IDX5 gene did not transmit the gene through the pollen.

1.3.3 Mucosal vaccination and choice of suitable antigens

Vaccination continues to be the most successful method of preventing infectious diseases in humans and livestock. Vaccination induces an immune response that is capable of limiting infection after exposure to the pathogen. Vaccines can be administered by a variety of means, including injection, intranasal or oral presentation (Bowersock and Martin, 1999). Pathogens responsible for the greatest burden of human disease make initial contact

with the human host at a mucosal site such as the respiratory, gastrointestinal, or genital tracts (Simmons et al, 1999). Oral vaccination targets the vaccine to the mucosal surface and results in stronger immune responses at the site of interaction of the pathogen and the host. Mucosal vaccines applied to the mucosal surface, may induce systemic antibodies and cellular immune responses as well as local immune responses (Ogra et al, 1980).

There are practical problems associated with oral vaccination. Oral vaccines must be protected during passage through the hostile environment (low pH, digestive enzymes) of the stomach en route to the Peyers patches of the small intestine where immune stimulation occurs (Bowersock and Martin, 1999). Strategies have been developed to protect vaccines from degradation in the harsh gastric and enteric environment, including encapsulation in liposomes, proteosomes, cochleates, virus-like particles, and immune-stimulating complexes (Simmons et al, 1999). Production of a vaccine in plant cells may protect it from the harsh stomach environment as it passes to the gut. Expression of antigens in transgenic plant tissues may provide an efficient bioencapsulation system. Viral and bacterial vectors for oral delivery of non-living antigens have been developed and tested in animals and humans for safety and immunogenicity (Tacket and Mason, 1999). This review will not be dwelling on that subject but would consider this aspect in production of a vaccine in maize.

Not all antigens are antigenic when delivered orally. One of the key problems with oral vaccinations is that non-living antigens delivered orally are in general poorly immunogenic, requiring multiple doses or large doses to stimulate modest immune responses, or a strong mucosal adjuvant to enhance the immune response (Bowersock and Martin, 1999). For this reason, the choice of antigen is critical. The most potent mucosal immunoadjuvants known to date are the labile toxin (LT) produced by some strains of

enterotoxigenic *E. coli* and cholera toxin (CT) produced by *Vibrio cholerae*, which are closely related. This makes them unique among soluble proteins, which are generally poorly immunogenic. The *E. coli* labile toxin (LT) and its derivatives were selected for their immunogenic properties, and are discussed below.

1.3.3.1 The *E. coli* heat labile toxin

LT is an 84-kilodalton polymeric protein composed of two major non-covalently associated immunologically distinct polypeptides designated (LT-A and LT-B). The A region (27 kDa) is responsible for increasing intracellular levels of cAMP and subsequent secretion of water and electrolytes into the small intestine, which is characteristic of the debilitating *E. coli* induced watery diarrhea (Spangler et al, 1992). LT-B is 55 kDa and consists of five non-covalently bound 11.6 kDa B subunits. These non-toxic B sub-units are responsible for binding the protein to receptors located on the surface of intestinal epithelial cells. Pentameric LT-B is responsible for binding of the toxin to the host cell receptor, G_{M1} (galactosyl-N-acetylgalactosaminyl-sialyl galactosyl glucosyl ceramide), which is commonly found on the surface of eukaryotic cells.

LT has two important characteristics for use in oral vaccination. LT-B has been shown to enhance the immune response to co-administered antigens and to abrogate the induction of oral tolerance to these antigens. Clements et al (1988) studied the effect of LT on oral tolerance non-target dietary antigens, and showed that prior exposure to the antigen reduces the effectiveness of LT in influencing tolerance and its ability to act as an adjuvant. LT was not able to abrogate tolerance once it was established. This implies that it can be safely used without the risk of an immune response being raised against normal dietary antigens

The molecular basis of the adjuvanticity and immunogenicity of LT appears to be poorly understood and is the subject of controversy in the literature. Both the toxic ADP-ribosylation activity of the LT-A subunit and the cellular toxin receptor, ganglioside, G_{M1}-binding properties of the LT-B-pentamer have been suggested to be involved (Dickinson and Clements, 1996). The general mechanism of holotoxin attachment and entry into the cell is that the B sub-unit binds to the G_{M1} gangliosides and internalizes the toxin into a vesicle from where it is transported to the trans-Golgi. There, the A and B sub-units dissociate and the A subunit is transported to the ER. From the ER the A1 subunit enters the cytosol where it interacts with the ADP ribosylation factor and it gets activated to initiate its toxic effects (Spangler, 1992). The characteristics of the LT subunits are important for the approach used in this dissertation and are therefore described below.

1.3.3.1.1 Role of the A subunit

The literature has different reports on the role of the A subunit. Some literature suggests that the A domain is the part of the molecule to which the adjuvanticity of these toxins should be attributed (Dickinson and Clements, 1996). In particular, the ADP ribosylation activity of the A subunit is thought to be critical. The A subunit can be cleaved into A1 and A2 subunits. The A2 fragment functions as an adapter between A and B subunits, and has a RDEL in LT (or KDEL in CT) that is associated with the retrieval of proteins from the trans-Golgi to the ER. This process is important in the delivery of the A1 subunit to the appropriate cellular component (Williams et al, 1999). ADP ribosylation by the A subunit probably contributes towards modulating the immune response where it is present. However, the A subunit is also thought to stabilize the conformation of the B subunit and to modulate vesicular trafficking through binding to K(R)DEL receptors by the A2 domain.

Mutants lacking ADP ribosylation activity have been shown to retain adjuvant activity (Pizza et al, 2001).

1.3.3.1.2 Role of the B subunit

There is disparity between studies of the effectiveness of B subunit alone as an adjuvant. Millar and co-workers (2001) showed that, contrary to previous opinion (reviewed by Dickinson and Clements, 1996), the non-toxic subunits of CT and LT, CT-B and LT-B, are potent oral immunogens, avoiding tolerance induction when administered mucosally and generating strong serum and mucosal responses (reviewed by Snider, 1995). They are arranged in a pentameric ring and contain five receptor-binding pockets for high avidity association with cellular G_{M1} gangliosides. Guidry et al (1997) examined the role of receptor binding in toxicity, immunogenicity and adjuvanticity of LT by comparing LT to a B-subunit receptor-binding mutant. The mutant was unable to serve as an effective oral adjuvant, and elicited negligible serum and mucosal immune responses. The conclusion from this work was that toxicity, immunogenicity and oral adjuvanticity of LT are dependent upon binding of the B subunit to ganglioside G_{M1}. The B subunit is thought to be the principal mediator of activity and its interaction with cell surface receptors triggers the key immunomodulatory events associated with adjuvant activity. LT-B can also serve as an immunologic carrier in traditional hapten-carrier configuration (Cebra et al, 1986). Other antigens conjugated to LT-B can therefore be immobilized for processing by the gut-associated lymphoid tissue (GALT).

1.3.3.1.3 Detoxification/Attenuation of LT

To facilitate its use as an adjuvant, researchers have attenuated the toxicity of LT

through genetic engineering of the A subunit (Yamamoto et al, 1997). Attempts to alter the toxicity of LT have focused on site-directed mutagenesis to change amino acids associated with the crevice where enzymatic activity is thought to occur. Progress appears to have been made towards detoxifying LT. To date, several mutant LT zootoxins have been developed, including LTK63, LTR72 and LTG192, among others (Pizza et al, 2001). In LTK63, Ser63 was changed to Lys through site directed mutagenesis. This mutation was expected to reduce the toxicity of LT by interfering with NAD^+ binding. Pizza et al (2001) showed that LTK63 was a strong and safe oral immunogen, and it consistently elicited higher antibody titers than bacterial LT-B. The mutant retained its adjuvant activity.

1.3.3.1.4. Potential of LT in design and production of mucosal vaccines

LT, its mutants and subunits have significant immunoregulatory potential as a means of preventing the induction of oral tolerance to specific antigens and as adjuvants for orally administered antigens. They also elicit the production of both serum IgG and mucosal IgA against antigens with which they are delivered. This makes these molecules potentially very useful in immunization programs against a variety of pathogens involving the oral administration of killed or attenuated agents or relevant virulence determinants of specific agents. Co-expression in transgenic crops of these adjuvant molecules with antigens from other economically important pathogens could further increase the utility of plant-based oral vaccines. LT-B and LT-A have been used as carrier molecules for other antigens whose small size or solubility limits their use as vaccines, or are not sufficiently immunogenic on their own. Conjugation of antigens to these subunits physically retains the antigen at high concentrations at the components of the immune system, stimulating an immune response. Virus neutralizing epitopes from the swine transmissible gastroenteritis virus (TGEV) were

selected for conjugation to the LT-B subunits.

1.3.3.2 Transmissible Gastroenteritis Virus (TGEV)

Transmissible gastroenteritis virus (TGEV) causes a highly contagious enteric disease characterized by vomiting, severe diarrhea and high mortality in young swine. In piglets younger than one week of age, mortality from this disease can be up to 100%, particularly under conditions of intensive farming. While progress made to understand the biology of the disease has led to improved management practices to control the disease, TGEV remains a major herd health problem in the United States. The economic losses due to this disease were conservatively estimated to be between 25 and 75 million dollars in the US (Paul *et al.* 1990) annually. Passive immunity is of primary importance in protecting newborn piglets against TGEV (Saif and Wesley, 1992). This type of immunity can be achieved by the induction of lactogenic immunity in the sow, which in turn can be induced by presentation of selected antigens to the immune system in the gut associated lymphoid tissues (Saif and Wesley, 1992). Pigs that survive a first infection are immune to subsequent infections of virus, probably due to local induction of secretory IgA. For this reason, vaccines that target the activation of the mucosal surface of the intestine are particularly attractive in the control of TGE and other mucosal diseases. Federally licensed vaccines are available, although they each have their limitations. The vaccines contain either inactivated virus or modified live attenuated virus. Inactivated viral vaccines are given intramuscularly in gilts and sows, and intraperitonially in baby pigs. The administration of both types of vaccine requires a lot of animal handling. With the oral vaccine approach, simply feeding the animals with transgenic plants expressing a suitable antigen can elicit a protective immune response. This approach potentially reduces the amount of animal handling required during animal vaccination.

TGEV is an enveloped, single stranded RNA virus. It belongs to the genus Coronavirus of the family Coronaviridae (Holmes and Lai, 1996). The virus consists of three structural proteins; the membrane protein, designated M, the phosphoprotein that encapsulates the viral RNA genome, designated N, and the large surface glycoprotein, designated S. The mature S protein forms the characteristic club-shaped peplomers or spikes, hence its designation. In this protein, four antigenic sites (A, B, C and D) have been defined (Delmas et al, 1986, 1990), all of them in the N-terminal half. Of these antigenic sites, site A is antigenically dominant, sites A and D and to a minor extent site B, have been involved in the neutralization of TGEV (Delmas, 1990). The S gene is therefore a suitable candidate for the development of recombinant subunit vaccines in baculovirus, plants and other expression systems (Callebautt et al, 1996; Shoup et al, 1997; Tuboly et al, 1994; Welter et al, 1996; Streatfield et al, 2001). This dissertation focused on the antigenic N-terminal half of the S protein common to TGEV and its mutant derivative, porcine respiratory coronavirus (PRCV) (Paul et al, 1990).

1.4 Research strategy

The potential of oral vaccination with a plant-produced antigen is beginning to be realized. Plant expressed antigens have been shown to be able to induce mucosal and systemic immunity when administered orally (Arakawa et al, 1998, Mason et al, 1998). A few of these vaccine candidates have been successfully tested in clinical trials or, where appropriate, in livestock.

There are several challenges to be addressed for the plant-based oral vaccine approach. Firstly, the transgenic plants must efficiently produce proteins that are antigenic,

that is, recognized by the host antibodies in response to authentic challenge. Secondly, the proteins produced must be immunogenic, eliciting antibodies when used for oral immunization and finally, the vaccines must generate a sufficient memory response to protect the test system from subsequent challenge with the disease causing agent (Artzen, 1997).

This dissertation focused on the expression of LT-B and a domain from the TGEV virus S protein. Expression of these two antigens in crops has been reported. Mason and co-workers (1998) used both an optimized and a non-optimized gene to express LT-B in transgenic potato. In both cases the CaMV 35S promoter regulated the gene. Their work showed that LT-B could be expressed in potato using either gene, but optimization of the LT-B gene resulted in increased amounts of LT-B protein in accumulation in potato tubers. The use of the ER retention signal SEKDEL also enhanced LT-B protein accumulation and up to 0.19% LT-B of total soluble protein was observed. They established the immunogenicity in mice and humans of the potato expressed LT-B, indicating that it was fully functional. Recently, Streatfield et al (2001, 2002) reported the expression of LT-B in maize, and established its immunogenicity in mice.

A number of attempts have been made to express the S gene of TGEV in transgenic plants (Welter et al, 1996; Streatfield et al, 2001) for use as a vaccine. Streatfield et al (2002) expressed the whole S protein in transgenic maize using a synthetic gene with codon usage optimized for expression in maize. They observed 0.01% S protein in maize kernel total soluble protein. In a pig feeding trial, they established that the maize-synthesized LT-B conferred some protection against challenge with TGEV when orally administered in piglets. However, the level of protein accumulation observed (0.01%) was low.

The strategy adopted in this study was to use the adjuvant and carrier molecule

properties of LT-B to deliver peptides carrying critical epitopes of TGEV to stimulate the immune system in the gut. The goal was to achieve high-level stable expression of the selected antigens in transgenic maize. Where possible, synthetic genes that had codon usage optimized for maize expression were used. The constitutive CaMV 35S promoter was used for gene expression in callus and the 27 kDa gamma zein promoter was used for kernel-specific expression of antigens. The bacterial and maize gamma zein signal peptides and the SEKDEL retention motif were used for subcellular targeting of the antigens in maize. TGE viral epitopes from the S protein were conjugated to subunits of LTK63, a non-toxic mutant derivative of LT, and expressed in transgenic maize callus. LTK63 was also used to determine whether higher order assembly was possible when separate subunits were expressed in maize as a control for the TGE/subunit fusion experiments. Immunogenicity of the maize synthesized proteins was determined in laboratory mice.

References

Arakawa, T, Chong, DKX, Merrit, JL, Langridge, WHR. 1998. Expression of Cholera toxin B subunit oligomers in transgenic potato plants. *Transgenic Research* **6**: 403-413.

Arntzen, CJ. 1997. High-tech herbal medicine: Plant based vaccines. *Nature Biotechnology* **15**: 221-222.

Bowersock, TL, Martin, S. 1999. Vaccine delivery to animals. *Advanced Drug Delivery Reviews* **38**: 167-194.

Bowman, CC, Clements, JD. 2001. Differential biological and adjuvant activities of Cholera toxin and *Escherichia coli* heat –labile enterotoxin hybrids. *Infection and Immunity* **69**: 1528-1535.

Callebaut, P, Enjuanes, L, Pensaert, M. 1996. An adenovirus recombinant expressing the spike glycoprotein of porcine respiratory coronavirus is immunogenic in swine. *Journal of General Virology*, **77**: 309-313.

Cebra, JJ, Fuhrman, JA, Lebman, DA, London, SD. 1986. Effective gut mucosal stimulation of IgA-committed B-cells by antigen. In Vaccines 86: New approaches to immunization. Developing vaccines against parasitic, bacterial and viral diseases. (Brown, F, Channok, R.M., Lerner, R.A., eds.), pp 129-133. Cold Spring Harbor Laboratory, New York.

Chikwamba RK, Cunnick J, Hathaway D, McMurray J, Mason H, Wang K. 2002. A functional antigen in a practical crop: LT-B producing maize protects mice against *Escherichia coli* heat labile enterotoxin (LT) and Cholera toxin (CT). *Transgenic Research,* **1122**: 1-15. In press.

Christou, P. 1996. Transformation technology. *Trends in Plant Sciences* **1**: 423-431.

Christensen, AM, Sharrock, RA, Quail, PH. 1992. Maize polyubiquitin genes: thermal perturbation of expression and transcript splicing and promoter activity following transfer to protoplasts by electroporation. *Plant Molecular Biology* **18**: 675-689.

Clements, JD, Hartzog, NM and Lyon, FL. 1988. Adjuvant activity of *Escherichia coli* heat-labile enterotoxin and effect on the induction of tolerance in mice to unrelated protein antigens. *Vaccine* **6**: 269-277.

Conrad, U and Fiedler U. 1998. Compartment-specific accumulation of recombinant immunoglobulins in plant cells: an essential tool for antibody production and immunomodulation of physiological functions and pathogen activity. *Plant Molecular Biology* **38**: 101-109.

Cramer, CL, Booth, JG, Oishi, KK. 1999. Transgenic plants for therapeutic proteins: liking upstream and downstream strategies. *Current Topics in Microbiology and Immunology* **240**: 95-118.

Daniell, H, Streatfield, SJ, Wycoff, K. 2001. Medical molecular farming: production of antibodies, biopharmaceuticals and edible vaccines in plants. *Trends in Plant Science* **6**: 219-226.

de Haan L, Feil IK, Verwej WR, Holtrop, M, Hol, WG, Agsteribbe, E, Wilschut, J. 1998. Role of GM1 binding in mucosal immunogenicity and adjuvant activity of the *Escherichia coli* heat labile enterotoxin and its B subunit. *Immunology* **94**: 424-430.

Delmas, B, Gelfi, M, Laude, H. 1986. Antigenic structure of transmissible gastroenteritis virus. II. Domains in the peplomer glycoprotein. *Journal of General Virology* **67**:1405-1418.

Delmas, B, Raaschaert, D, Godet, M, Gelfi, J, Laude, H. 1990. Four major antigenic sites of the coronavirus transmissible gastroenteritis virus are located on the amino terminal half of the spike glycoprotein S. *Journal of General Virology* **71**: 1313-1323.

Dickinson, BL, Clements, JD. 1995. Dissociation of *Escherichia coli* heat labile enterotoxin adjuvanticity from ADP-ribosyltransferase activity. *Infection and Immunity* **63**: 1617-1623.

Dickinson, BL, Clements, JD. 1996 Use of *Escherichia coli* heat labile toxin as an oral adjuvant. *In:* Kiyono, H, Ogra, PL and McGhee (eds), *Mucosal Vaccines*, Academic Press, pp73-87.

Gribskov M, Devereux J, Burgess RR. 1984. The codon preference plot: Graphic analysis of protein coding sequences and prediction of gene expression. *Nucleic Acids Research* **12**: 539-549.

Guidry, JJ, Cardenas, L, Cheng, E, Clements, JD. 1997. Role of receptor binding in toxicity, immunogenicity and adjuvanticity of *Escherichia coli* heat labile enterotoxin. *Infection and Immunity* **65**:4943-4950.

Haq, TA, Mason, HS, Clements, JD, Arntzen, CJ. 1995. Oral Immunization with a recombinant bacterial antigen produced in transgenic plants. *Science* **268**: 714-716.

Hirst, TR, Hardy, SJS and Randal, LL. 1983. Assembly in vivo of enterotoxin from Escherichia coli: formation of the B subunit oligomer. *Journal of Bacteriology* **153**: 21-26.

Holmes, KV, Lai, MMC. 1996. Coronaviridae: The viruses and their replication. In Fields, B.N., Knipe, D.M., Howley, P.M. et al, (eds). Fields Virology, Third Edition. Lippincott-Raven Publishers, Philadelphia.

Hood EE, Witcher DR, Maddock S, Meyer, T, Baszczynski, C, Bailey, M, Register, J, Marshall, L, Bond, D, Kulisek, E, Kusnadi, A, Evangelista, R, Nokolov, Zivko, Wooge, C, Mehogh, RJ, Herman, R, Kappel, WK, Ritland, D, Li, CP, Howard, JA. 1997. Commercial production of avidin from transgenic maize: characterization of transformant, production, processing, extraction and purification. *Molecular Breeding* **3**: 291-300.

Kusnadi, AR, Nikolov, ZL, Howard, JA. 1997. Production of recombinant proteins in transgenic plants: Practical considerations. *Biotechnology and Bioengineering* **56**: 473-484.

Levine, MM, Kaper, JB, Black, RE, Clements, ML. 1993. New knowledge on pathogenesis of bacterial enteric infections as applied to vaccine development. *Microbiology Reviews* **47**: 510-550.

Marks, MD, Lindell, JS, Larkins, BA. 1985. Quantitative analysis of the accumulation of zein mRNA during endosperm development. *Journal of Biological Chemistry* **260**: 16445-16450.

Mason, HS, and Arntzen, CJ. 1995. Transgenic plants as vaccine production systems. *Trends in Biotechnology* **13**: 388-392.

Mason, HS, Ball, JM, Shi, JJ, Jiang, X, Estes, MK, Arntzen, CJ. 1996. Expression of the Norwalk virus capsid protein in transgenic potato and its oral immunogenicity in mice. *Proceedings of the National Academy of Science USA* **93**: 5335-5340.

Mason, HS, Haq, TA, Clements, JD, Arntzen, CJ. 1998. Edible vaccine protects mice against *Escherichia coli* heat labile enteretoxin (LT): potatoes expressing a synthetic LT-B gene. *Vaccine* **16**: 1336-1343.

Mason, HS, Lam, DM-K, Arntzen, CJ. 1992. Expression of the herpatitis B surface antigen in transgenic plants. *Proceedings of the National Academy of Science USA* **89**: 11745-9.

Matzke, AM, Matzke, AJM 1995. How and why do plants inactivate homologous transgenes? *Plant Physiology* **107**: 679-685.

McGarvey, PB, Hammond, J Dienelt, MM, Hooper, DC, Fu, ZF, Dietzschold, B, Koprowski H, Michaels, FH. 1995. Expression of the rabies virus glycoprotein in transgenic tomatoes. *Bio/Technology* **13**: 1484-7.

Millar DG, Hirst T, Snider DP. 2001. *Escherichia coli* heat labile enterotoxin B sub-unit is a more potent mucosal adjuvant than its closely related homologue, the B sub-unit of Cholera toxin. *Infection and Immunity* **69**: 3476-3482.

Moffat, AS. 1995. Exploring transgenic plants as a new vaccine source. *Science* **268**: 658-660.

Munro S. Pelham HRB. 1987. A C-terminal signal prevents secretion of luminal ER proteins. *Cell* **48**: 899-907.

Nagy, F, Odell, JT, Morelli, G, Chua, NH. 1980. Properties of expression of the CaMV 35S promoter from CaMV in transgenic tobacco plants. In: Zaitlin, M, Day,P, Hollaender A, (eds) Biotechnology in Plant Science: Relevance to agriculture in the Eighties. Pp. 227-235. Academic Press, Orlando, FL.

Ogra, PL, Fishaut, M, Gallagher, MR. 1980. Vaccination via the mucosal routes. *Reviews of Infectious Diseases* **2**:352-369.

Paul, PS, Zhu, XL, and Vaughan, E. 1988. Current Strategies for the Development of Efficacious Vaccines for Transmissible Gastroenteritis in Swine. In: Proceedings of the 92nd Annual Meeting of the US Animal Health Association, Little Rock, Arkansas, pp. 429 - 443.

Paul, PS, Zhu, XL, and Vaughan, E 1994. Significance of Porcine Respiratory Coronavirus Infection. *Continuing education for the practical Veterinarian* **16**: 1223-1234.

Pen J, Sijmons PC, Van Ooijen AJJ, Hockema A. 1993. Protein production in transgenic crops: Analysis of plant molecular farming. In: Industrial Crops Production. pp. 241-250. Elsevier, Amsterdam.

Pizza, M, Giuliani, MM, Fontana, MR, Monaci, E, Douce, G, Dougan, Mills, KHG, Rappuoli, R, Del Giudice, G. 2001. Mucosal vaccines: non-toxic derivatives of LT and CT as mucosal adjuvants. *Vaccine*: **19**: 2534-2541.

Saif L.J. and R.D. Wesley. 1992. Transmissible Gastroenteritis. In: A.D. Leman, B.E. Straw, W.L. Mengeling, S. D'Allaire and D.J.Taylor (Eds.), Disease of Swine, pp 362-386. Wolfe Publishing Ltd.

Sangtong, V, Moran, DL, Chikwamba, RK, Wang, K, Woodman-Clikeman W, Long, MJ, Lee, M, Scott, MP. 2002. Expression and inheritance of the wheat Glu-1DX5 gene in transgenic maize. *Theoretical and applied Genetics (TAG).* In press.

Shoup, D.I, Jackwood, D.J. and Saif, L.J. 1997. Active and passive immune responses to transmissible gastroenteritis virus (TGEV) in swine inoculated with recombinant baculovirus-expressed TGEV spike glycoprotein viruses.

Simmons, CP, Ghaem-Magami, M, Petrovska, L, Lopes, L, Chain, BM, Williams, NA, Dougan, G. 2001. Immnomodulation using bacterial enterotoxins. *Scandanavian Journal of Immunology* **53**: 218-226.

Snider, DP. 1995. The mucosal adjuvant activities of ADP-ribosylating bacterial enterotoxins. *Reviews in Immunology* **15**: 317-348.

Stachelin, LA, Moore, I. 1995. The plant Golgi apparatus: Structure, functional organization and trafficking mechanisms. *Annual Reviews Plant Physiology and Plant Molecular Biology* **46**: 261-288.

Streatfield SJ, Jilka JM, Hood EE, Turner DD, Bailey MR, Mayor JM, Woodard SL, Beifuss KK, Horn ME, Delaney DE, Tizard IR, Howard JA. 2001. Plant based vaccines: Unique advantages. *Vaccine* **19**: 2742-2748.

Streatfield SJ, Mayor, JM, Barker, DK, Brooks, C, Lamphear BJ, Woodard SL, Turner DD, Beifuss KK, Horn ME, Delaney DE, Tizard IR, Jilka JM, Hood EE, Howard JA. 2002. Development of an edible subunit vaccine in corn against enterotoxigenic strains of *Escherichia coli. In Vitro Cellular and Developmental Biology-Plant* ***38****:* 11-17.

Tacket, CO, and Mason, HS. 1999. A review of oral vaccination with transgenic vegetables. *Microbes and Infection* **1**: 777-783.

Tacket, CO, Mason, HS, Lasonsky, G, Clements, JD, Levine, MM, Arntzen, CJ. 1998. Immunogenicity in humans of a bacterial antigen delivered in a transgenic potato. *Nature Medicine* **4**:607-609.

Torres, JM, Sanchez, CM, Sune, C, Smerdou, C, Prevec, L, Graham, F, Enjuanes, L. 1995. *Virology* **213**:503-516.

Tuboly, T, Nagy, E, Dennis, JR, Derbyshire. 1994. Immunogenicity of the S protein of transmissible gastroenteritis virus expressed in baculovirus. *Archives of Virology* **137**: 55-67.

Welter, LM, Mason, HM, Lu, W, Lam, DM-K, Welter, MW. 1996. Effective immunization of piglets with transgenic potato plants expressing a truncated TGEV S protein. Vaccines: New Technologies and Applications. Cambridge Healthtech Institutes.

Yamamoto, T, Tamura, T, Ryoji, M, Kaji, A, Yokota, T, Takano, T. 1982. Sequence analysis of the heat-labile enterotoxin subunit B gene originating in human enterotoxigenic *Escherichia coli. Journal of Bacteriology* **152**: 506-509.

Zhong GY, Peterson D, Bailey M, Witcher DR, Register J, Bond D, Li CP, Marshall L. Kulisek E, Ritland D, Meyer T, Hood EE, Howard JA. 1999. Commercial production of aprotinin in transgenic maize seed. *Molecular Breeding* **5**: 345-356.

CHAPTER 2

Expression of a synthetic *E. coli* heat-labile enterotoxin B sub-unit (LT-B) in maize

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Abstract

We have produced the B subunit of the enterotoxigenic *Escherichia coli* (ETEC) heat-labile enterotoxin (LT-B) in transgenic maize seed. LT-B is a model antigen that induces a strong immune response upon oral administration and enhances immune responses to conjugated and co-administered antigens. Using a synthetic LT-B gene with optimized codon sequence, we examined the role of promoters and the SEKDEL endoplasmic reticulum retention motif in LT-B accumulation in callus and in kernels. Two promoters, the constitutive CaMV 35S promoter and the maize 27 kDa gamma zein promoter, which directs endosperm-specific gene expression in maize kernels, regulated LT-B expression. Ganglioside-dependent ELISA analysis showed that using the constitutive promoter, maximum LT-B level detected in callus was 0.04% LT-B in total aqueous-extractable protein (TAEP) and 0.01% in R_1 kernels of transgenic plants. Using the gamma zein promoter, LT-B accumulation reached 0.07% in R_1 kernels. The SEKDEL resulted in increased LT-B levels when combined with the gamma zein promoter. We monitored LT-B levels under

greenhouse and field conditions over three generations. Significant variability in gene expression was observed between transgenic events, and between plants within the same event. A maximum of 0.3% LT-B in TAEP was measured in R_3 seed of a transgenic line carrying CaMV 35S promoter/LT-B construct. In R_3 seed of a transgenic line carrying the gamma zein promoter/LT-B construct, up to 3.7% LT-B in TAEP could be detected. We conclude that maize kernels can be used as a production system for functional antigens.

Key words:

Functional antigen, gene expression, LT-B, *Zea mays*

Abbreviations:

BSA, bovine serum albumin; **CaMV 35S**, Cauliflower Mosaic Virus 35S RNA Promoter; **EDTA**, ethylene-diamine tetraacetic acid; **ELISA**, enzyme-linked immunosorbent assay; G_{M1}, galactosyl-N-acetylgalactosamyl-sialyl-galactosylglucosyl ceramide; **LT-B**, heat-labile toxin from *E. coli*; **PBS**, phosphate-buffered saline; **PMSF**, phenylmethanesulfonyl fluoride; **SDS-PAGE**, sodium dodecyl sulfate polyacrylamide gel electrophoresis; **Tris**, Tris hydroxymethyl aminomethane.

Introduction

Recombinant DNA technology and advances in plant transformation have expanded the utility of plants beyond the traditional uses of food, feed and fiber. Plants are now used as a source of proteins for industrial, pharmaceutical, and other purposes (Krebbers et al., 1993; Pen et al., 1993; Austin et al., 1994). Production of recombinant proteins in transgenic plants is emerging as a competitive and safe alternative to the traditional protein expression

systems. This is particularly relevant for the production of monoclonal antibodies and antigens for use as vaccines in humans and animals. A major advantage of producing antigens for this purpose in a crop like maize is that plant-derived antigens are free from human and animal pathogens normally associated with vaccines produced in conventional mammalian cell culture systems. To use plants as an efficient system for antigen production, a number of issues need to be considered. Transgenic lines should have high expression levels of the gene of interest in appropriate tissues. In addition, the recombinant proteins produced *in planta* should be have structural integrity and be functionally active. Moreover, transgenic plants should be fertile and transmit and predictably express the transgene over generations. Novel protein production in plants has been reported in potato, tobacco, and Arabidopsis. Advances in maize transformation have allowed us to investigate the possibility of using this crop as a source of antigens to be used as oral vaccines.

In this study we report the production of a functional *Escherichia coli* (*E. coli*) heat labile enterotoxin B sub-unit (LT-B) in maize. LT-B is part of the heat-labile toxin (LT) produced by enterotoxigenic strains of *E. coli*, a leading cause of diarrhea in developing countries (Spangler, 1992). The bacterium is ingested in contaminated food or water and colonizes the gut, where it secretes toxins, including LT. LT is an 84-kilodalton (kDa) oligomeric protein composed of two major noncovalently associated immunologically distinct peptides designated LT-A and LT-B. The A peptide (27 kDa) is responsible for the diverse biological effects of the toxin. LT-B is a 55 kDa homopentamer of 11.6 kDa peptides responsible for binding of the toxin to the host cell receptor G_{M1} (galactosyl-N-acetylgalactosaminyl-sialyl galactosyl glucosyl ceramide), which is found on the surface of eukaryotic cells. Adjuvants are substances that, either alone or linked to protein antigens,

can stimulate mucosal responses and therefore significantly enhance the potency of vaccines in general. They are particularly important in local vaccination with nonreplicating antigens, which can be ineffective because they are poorly immunogenic. LT-B has been shown to be an excellent oral adjuvant, stimulating immune responses against co-fed antigens, enhancing their value as vaccines (Millar et al., 2001). LT-B can serve as an immunologic carrier in traditional the hapten-carrier configuration as well (Spangler, 1992). Other antigens conjugated to LT-B can be immobilized for processing by the gut-associated lymphoid tissue (GALT) because of LT-B's affinity to G_{M1} gangliosides on the surface of the intestinal mucosa.

LT-B produced in potato and maize has been shown to be immunogenic in mice (Mason et al., 1998; Streatfield et al., 2001; Chikwamba et al., 2002) and humans (Tacket et al., 1998). This protein is an ideal model for an oral vaccine, and its adjuvant properties make it attractive for use in co-expression or co-administration with other plant-synthesized or conventional vaccines. We have generated transgenic maize plants expressing LT-B in kernels using several constructs. We investigated the role of promoters and the SEKDEL amino acid motif, an endoplasmic reticulum-retention signal, in the accumulation of LT-B in transgenic maize. A high level of expression is important for meeting dosage requirements for plant-based biologics. We also examined the expression of LT-B over three generations in the greenhouse and the field. Our data indicate that maize kernels, a key ingredient in livestock feed, can be used to produce functional antigens for use as feed-based vaccines.

Materials and methods

Constructs

A plant codon-optimized LT-B subunit gene (Mason et al., 1998) was cloned into four different constructs (Figure 1). Construct pTH210 (Mason et al., 1998, Figure 1A), has the LT-B gene regulated by the CaMV 35S promoter, the tobacco etch virus (TEV) leader as a translational enhancer (Gallie et al., 1995), and the soybean vegetative storage protein (VSP) terminator (Rhee and Staswick, 1992) in the 3'terminus. Transgenic maize calli and plants carrying this construct were designated P51. Construct pTHK210 (Mason, unpublished, Figure 1B) is identical to pTH210, except a SEKDEL (Ser-Glu-Lys-Asp-Glu-Leu) encoding sequence is included in the C-terminus of the LT-B encoding sequence. Plants carrying this construct were designated P65. In construct pRC4 (Figure 1C), the CaMV 35S promoter was replaced by the maize 27 kDa gamma zein promoter (Marks et al., 1985). To construct pRC4, a 1.15 kb *Xho* I-*Eco*R I fragment including the TEV 5', the synthetic LT-B coding sequence and the VSP terminator from pTH210 was cloned downstream of the gamma zein promoter in the vector pUC19. Plants carrying this construct were designated P77. Construct pRC4-1 (Figure 1D) is identical to pRC4 except it carried the LT-B gene with the SEKDEL motif in its C-terminus. Plants with this construct were designated P112. This construct was made by subcloning the 1.15 kb *Xho* I and *Eco*R I fragment from plasmid pTHK210 downstream of the maize 27 kDa gamma zein promoter in a pUC19 vector. In all cloning work, the resultant plasmids were sequenced to ensure correct orientation and fidelity of ligation junctions. DNA for maize transformation was obtained

using the Qiagen (Qiagen GmbH, Germany) Maxiprep kit according to the manufacturer's instructions.

Maize transformation

Maize transformation was conducted by the Iowa State University Plant Transformation Facility using the procedure described by Frame et al. (2000). Briefly, embryogenic calli generated from immature embryos of the Hi-II genotype were transformed using microprojectile bombardment. The constructs carrying the LT-B gene were co-bombarded with pBAR184 (Frame et al., 2000), a plasmid that carries the maize ubiquitin promoter-*bar* gene cassette as a selectable marker conferring resistance to the herbicide bialaphos. The bialaphos-resistant calli were analyzed by PCR, Southern blot, and Northern blot and ELISA analyses as described below. Calli of transgenic events were regenerated and brought to maturity in the greenhouse. R_1 seed were obtained by out-crossing, with the transformants as female parents and untransformed inbred lines B73 and/or Hi-II as male parents. Seeds from LT-B-expressing events were planted to give R1plants.

PCR analysis

Transgenic calli were initially selected on the basis of resistance to bialaphos, and the presence of the LT-B gene was confirmed by PCR analysis. The LT-B gene was amplified from 10 to 100 ng of total genomic DNA extracted from 0.1-0.25 g of callus as described by Chikwamba et al. (2002). Callus DNA was extracted in 400 µl extraction buffer [200 mM Tris-Cl (pH 7.5), 250 mM NaCl, 25 mM EDTA, and 0.5% SDS (w/v)], followed by extraction in an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1, V:V:V). PCR

primers and conditions, used as described (Chikwamba et al., 2002), were the same in all cases: LTB-PCRF1 (5'-atc gat aca aaa caa acg aat ctc aag c-3') and LTB-PCRR1 (5'-cca tgg cgt gga ttt tat gac att tta t-3'). These 28 bp primers amplify an 842 bp fragment from the TEV leader at the 5' end to part of the VSP terminator at the 3' end, including the LT-B-coding sequence.

Southern blot analysis

Southern blot analysis was undertaken on DNA from leaf tissue of young putative transformants or R_2 plants to confirm transformation and to estimate the copy number of the LT-B gene. Genomic DNA was extracted from leaves of maize plants using a cetyltrimethylammonium bromide (CTAB) protocol (Murray and Thompson, 1980). Ten micrograms of leaf genomic DNA was digested with restriction enzyme *Hin*d III for P51 and P65, and *Nco* I for P77 and P112, at 37°C overnight and separated on a 0.8% agarose gel. Linearized DNA gel blot analysis (Sambrook et al., 1989) was conducted on DNA samples using a ^{32}P-labeled LT-B gene fragment excised from pTH210 using the enzymes *Nco* I and *Kpn* I as probe (Figure 1).

Northern blot analysis

Northern blot analysis was conducted using the leaf tissue of selected transgenic plants. Total RNA was extracted from leaf tissue of R_0 and R_1 plants using Trizol reagent (Gibco Life Sciences, Rockville, MD, USA) according to the manufacturer's directions. Twenty micrograms of total RNA was separated on a 1% agarose gel under RNAse-free denaturing conditions and then blotted onto a nitrocellulose membrane. The LT-B gene

fragment was also used as a probe in the hybridization procedure as described for Southern blot analysis.

Evaluation of protein production

Evaluation of protein expression was carried out in callus and maize kernels. About 0.25 g of fresh callus was homogenized in 500 µl of protein extraction buffer [25 mM Sodium phosphate (pH 6.6) 100 mM NaCl, 0.5% Triton X-100 (v/v), 10 µg/ml leupeptin (w/v)]. Two methods were used for processing dry maize kernels, one manual and the other partially mechanized. In the manual procedure, individual kernels were partially crushed with a mortar and pestle, frozen in liquid nitrogen, and then ground to a fine powder. In the mechanized method, individual kernels and a single ball bearing (3/8 inch, Dynasteel) were placed in a polycarbonate tube (1/2 x 2 inches, Spex CertiPrep, Metuchen, NJ, USA). The tubes were shaken in a Spex CertiPrep GenoGrinder for 5-10 minutes at 1400 rpm, depending on the hardness of the kernels, until the kernels were reduced to a fine powder.

Protein extraction buffer was added at a ratio of 10 µl per mg of dry, finely ground kernel tissue in an Eppendorf tube; 30 to 50 µg of tissue was used per sample. The samples were placed on a vortex shaker for 30 to 60 minutes for complete resuspension and allowed to sit on the bench for another hour, after which they were centrifuged at 14,000 rpm in a microcentrifuge for 15 minutes at room temperature. One hundred microliters of the supernatant (50 µl per well, 2 replications/sample) was used for ganglioside-dependent ELISA analysis as previously described (Mason et al., 1998). Total soluble protein concentrations were determined by the Bradford Assay (Bradford, 1976) with the BioRad

(Hercules, CA, USA) protein dye concentrate using a standard curve derived from bovine serum albumin (BSA).

To identify kernels expressing LT-B for planting for the next generation, individual kernels from each ear (two ears per event) were analyzed by a partial destruction method (Sangtong et al., 2001). With the use of a fine drill and without damaging the embryo, part of the endosperm from each kernel was removed for ELISA analysis. Protein was extracted from the endosperm as described for whole kernels. Kernels expressing LT-B were planted to give rise to the subsequent generation.

Quantitation of LT-B expression in maize

LT-B expression in maize was determined using ganglioside-dependent ELISA (Mason et al., 1998; Chikwamba et al., 2002). Reagents and antisera for the ELISA were obtained from Biogenesis, Inc . (Kingston, NH, USA) and KPL (Gaithersburg, MD, USA). Volumes of 50 µl were used throughout all ELISA assays unless otherwise specified. Wells were washed three times between each step using 300 µl of phosphate-buffered saline Tween-20 [PBST; 0.01 M Na_2HPO_4, 0.003 M KH_2PO_4, (pH 7.2), 0.1 M NaCl, 0.05% Tween-20 (v/v)]. Each step of the ELISA was carried out at 37°C throughout the process unless otherwise specified. Briefly, LT-B from plant extracts was captured in microtiter plates (Costar 3590, Fisher Scientific, PA, USA) coated with Type III G_{M1} gangliosides (1.5 µg/well) from bovine brain (G2375, Sigma, St Louis, MO, USA), dissolved in sodium carbonate coating buffer [15 mM Na_2CO_3, 35 mM $NaHCO_3$, 3 mM NaN_3, (pH9.6)] for 1 hour at room temperature. The plates were blocked to prevent nonspecific antibody binding with 5% dry milk (w/v, DIFCO, Becton Dickinson, MD, USA) for one hour at room

temperature. The LT-B in crude extracts was captured for one hour at 37°C or 4°C overnight, followed by incubation with goat anti-LT-B antiserum (1:1500 dilution, Biogenesis, Kingston, NH, USA) at 37°C for one hour. Rabbit anti-goat alkaline phosphatase conjugate [1:2500 dilution in 1% dry milk (w/v) in PBS] was added, and the plate incubated for one hour. This was followed by addition of phosphatase substrate, which consisted of 1 mg/ml ρ-nitrophenyl phosphate (ρNPP, Sigma) in alkaline phosphatase buffer [0.1M Tris, (pH 9.5), 0.05M $MgCl_2$, 0.1M NaCl]. Absorbance at 405 nm was immediately measured spectrophotometrically over a two-hour period in a Dynatech MRX Plate Reader (Dynex Technologies, VA, USA). Sample wells were blanked against nontransgenic maize protein extracts, and all measurements were performed in duplicate. Raw ELISA data were converted to percentage of LT-B of total aqueous extractable protein by reference to an ELISA standard curve constructed using purified bacterial LT-B (kindly provided by John Clements, Tulane University, New Orleans, LA, USA).

Screening of transgenic plants in field

To identify transgene-expressing plants in the field, the segregating R_2 plants were sprayed with 200 mg/L Liberty®(w/v) with 0.05% Tween-20 (v/v) two weeks post-emergence. The gene expressing plants were either self pollinated or crossed with their siblings. The segregation ratio of herbicide resistance was approximately 3:1 (resistant: susceptible), as expected, and the herbicide-resistant plants were tagged for identification. Both tassels and ears were bagged, and because plants were both male- and female-fertile, they were, in most cases, self-pollinated to produce R_3 seed.

Data analysis

To determine the expression level for each ear, twenty kernels per ear were analyzed for LT-B expression, and an average expression level was obtained from only the LT-B positive kernels. The data are presented as the mean ± standard deviation. For P51 and P77 R_3 kernels, ELISA analyses were carried out on eight ears, 20 whole kernels per ear as described. For each ear, 10 values of 10 randomly selected LT-B positive kernels and used to determine the mean expression per ear. Ten kernels were picked for convenience of statistical analysis as each segregating ear had a different number of gene expressing kernels. Statistical analysis (analysis of variance) were carried out using the package SAS (The SAS Institute Inc., Cary, NC, USA).

Results

Generation of LT-B-producing transgenic maize plants

The constructs used for maize transformation are shown in Figure 1. The LT-B gene in all constructs was synthesized with a codon bias for expression in potato and maize (Mason et al., 1998). In constructs pTH210 and pTHK210, the CaMV 35S promoter, the TEV enhancer, and the VSP terminator regulated the LT-B gene. In construct pTHK210, a DNA sequence encoding the SEKDEL amino acid motif, which binds to the SEKDEL receptor in the endoplasmic reticulum (Munro and Pelham, 1987), was included in the C-terminus of the LT-B coding sequence. This motif has been shown to enhance the expression of a non-optimized LT-B gene in transgenic potato (Haq et al., 1995). Transgenic maize events carrying these two constructs were designated P51 and P65, respectively. In

constructs pRC4 and pRC4-1, the maize 27 kDa gamma zein promoter replaced the CaMV 35S promoter in pTH210 and pTHK210, respectively. PRC4-1 has the SEKDEL motif in the C-terminus of LT-B. Transgenic events carrying these two constructs were designated P77 and P112, respectively (Figure 1).

PCR analyses of the LT-B transgene insertions were carried out on herbicide-resistant calli. The co-transformation rate for the selectable marker gene *bar* and the gene of interest LT-B in all four constructs ranged from 58 % to 89 %, with an average of 77%. Southern blot analysis was carried out on genomic DNA isolated from leaf tissue of putative transformants. Results of representative events from construct pTH210 (P51) and pRC4-1 (P112) are shown in Figures 2A and B, respectively. Genomic DNA was digested with restriction enzymes that cut once within the construct; *Hind* III was used for P51 (Figure 2A) and P65 (data not shown). *Eco*R I and *Nco* I were used for P77 (Chikwamba et al., 2002) and P112 (Figure 2B), respectively. Results showed integration of the LT-B gene cassette into the maize genomic DNA. Each event had a unique insertion pattern, and plants from the same event had similar transgene insertion patterns (P51-20, Figure 2A). Most of the events were shown to contain multiple copies of the transgene. Estimated copy number ranged from two to more than, as is typical of plants transformed via microprojectile bombardment (Christou, 1996).

Figure 3 shows the Northern blot analysis results on representative events of P51. A transcript with ~700 nucleotides hybridized with the LT-B probe and could be detected in RNA from leaf of transgenic plants that had been regenerated on the basis of herbicide resistance, PCR analysis, and callus expression of LT-B (see below). Different levels of mRNA expression were observed, and Event #23 had no detectable LT-B RNA, despite

having been shown to contain multiple copies of the transgene (data not shown). These results showed the LT-B gene was being transcribed in maize leaf tissue in transgenic plants carrying pTH210.

Constitutive expression of LT-B protein

Table 1 summarizes the level of LT-B protein expression in callus under the constitutive promoter CaMV 35S in maize. LT-B was quantified using the ganglioside-dependent ELISA procedure (Haq et al., 1995; Chikwamba et al., 2002). Because ganglioside binds only multimeric LT-B protein, these positive ELISA results indicate that the protein was being properly assembled in maize tissue. Of the 54 independent P51 transgenic events, 42 (78%) were shown to express LT-B at levels less than 0.005% of total aqueous-extractable protein (TAEP). Nine events (17%) had LT-B protein levels between 0.005 and 0.01% in TAEP. Only three (6%) of the callus lines had LT-B protein level above 0.01% in TAEP. A similar range of LT-B expression was observed for P65 transgenic events. Of 51 events tested, five callus events (10%) had LT-B levels higher than 0.01% of TAEP. The majority of events (90%) had LT-B levels less than 0.01% of TAEP. Maximum expression for both constructs (Events P51-62 and P65-69) was 0.04% LT-B of TAEP. While the number of events with LT-B protein above 0.01% of TAEP, the SEKDEL microsomal retention signal included in the construct pTHK210 (P65) did not substantially increase LT-B protein accumulation in maize callus compared with P51 events (Table 1).

The level of LT-B protein in young leaf tissue of P51 and P65 plants was also analyzed. In general, LT-B levels in transgenic leaf tissue were consistent with levels in corresponding callus tissue, with a few exceptions in which the LT-B level declined in leaf

tissue (data not shown). We also observed that plant regeneration, vigor, and seed set were generally poor in both P51 and P65 events that showed high expression of LT-B protein in callus (data not shown).

For determination of the level of LT-B protein in seed, R_1 kernels from each event were homogenized manually or mechanically and assayed individually. Based on the levels of total protein extracted from kernels using these two methods, there were no differences in the protein yield between these two methods. A total of three P51 events and nine P65 events were tested. Poor seed set was a limiting factor for P51 events. Most events tested showed less than 0.005% LT-B in TAEP in whole seed. The maximum level of LT-B (0.01% LT-B in TAEP) was detected in one P51 event (P51-62) and one P65 event (P65-69) (data not shown). When embryo and endosperm tissue from a single kernel were analyzed separately, the LT-B protein was detected primarily but not exclusively in the embryo as expected (data not shown).

Kernel-specific expression of LT-B

Transgenic maize plants carrying the constructs pRC4 and pRC4-1 (Figure 2C and D) were also produced. Since the LT-B gene in these constructs was regulated by the 27 kDa gamma zein promoter, a kernel-specific promoter, LT-B expression was not detected in callus tissues as expected (data not shown). A total of 19 P77 events and 20 P112 events were generated. For analysis of the LT-B protein levels in R_1 kernels, proteins from 20 kernels per ear representing each event were extracted separately and assayed for LT-B expression by ELISA. Data from all LT-B positive kernels in each ear were pooled to determine the mean and standard deviation (SD) for each ear.

Levels of LT-B accumulation in P77 (one ear per event) and P112 (two ears per event) are presented in Figures 4 and 5, respectively, and summarized in Table 2. Of 19 LT-B-expressing P77 events, 11 (58%) had LT-B protein levels higher than 0.01% of TAEP (Figure 4). Two events (P77-2 and P77-3) had LT-B levels of up to 0.07%.

P112 transgenic events showed the highest level of LT-B accumulation in R_1 seeds (Figure 5). P112 events carry the construct pRC4-1, which included the endoplasmic reticulum-retention signal sequence SEKDEL in the C-terminus of LT-B gene. All 15 P112 events tested had LT-B levels of 0.01% or higher (Table 2). In addition, six events had LT-B levels higher than 0.1%. One event (P112-44) accumulated up to 0.9% of LT-B in TAEP, about 13-fold higher than the highest-expressing P77 R_1 event (P77-3). This is in contrast to the observations for transgenic events P51 and P65, in which the SEKDEL motif did not increase the LT-B protein level in callus tissue. Inclusion of the SEKDEL motif in the LT-B gene under the regulation of the seed-specific promoter greatly enhanced the LT-B level in P112 events.

Plant performance comparison

Compared to transgenic maize plants carrying CaMV 35S promoter/LT-B constructs (P51 and P65), plants carrying zein promoter/LT-B constructs (P77 and P112) were more vigorous in overall performance. Table 3 summarizes the seed set from transgenic lines P51, P65, P77, and P112. The seed set of P51 was the poorest; only 23% of events set more than 50 kernels per event. For P65, although the level of assembled LT-B protein did not increase, the fertility of transgenic plants was markedly increased over that of P51 plants. Sixty-four percent of events set more than 50 seeds. Both P77 and P112 had good fertility.

Seventy-four percent of P77 events and 55% of P112 events produced more than 50 kernels per event, respectively. The transgene segregated 1:1 or 3:1 as expected in most cases.

Evaluation of LT-B protein levels in R_2 and R_3 seeds

To evaluate stability of LT-B gene expression under control either of a constitutive promoter or a seed-specific promoter over generations, further analysis was performed using one event of P51 (Event P51-62) and eight events of P77 (Events P77-2, 3, 7, 9, 10, 11, 17, and 18) (Tables 4 and 5).

P51-62 had the highest LT-B accumulation (0.01% TAEP) in R_1 seed. Five plants of this event were grown in the greenhouse, and two were successfully self-pollinated to produce R_2 seeds. The LT-B levels measured in R_2 seeds had a fourfold increase compared with R_1 kernels, with 0.04% LT-B of TAEP (data not shown). P51-62 R_2 seeds were grown in the field in the summer of 2001. Transgene-expressing plants were first identified by resistance to the herbicide bialaphos sprayed two weeks post emergence and subsequent self-pollination to produce R_3 seeds. LT-B expression was tested in eight ears (20 kernels per ear) of event P51-62 (Table 5). Mean expression was obtained from 10 randomly selected LT-B expressing kernels. The levels of LT-B varied greatly between ears derived from the same event. These values ranged from 0.026% (ear #8) to 0.28% (ear #1) with an average of 0.14% in TAEP. Within the same ears, large standard deviation values indicate differences in expression from kernels on the same ear. The LT-B level in R_3 ear #7 (0.28%) represented a further six-fold increase when compared with the level in the R_2 seed (0.04%).

Eight P77 events with LT-B levels ranging from 0.017% (Event P77-10) to 0.069% (Event P77-3) were followed to the R_2 generation. Two to three plants representing each

event were self-pollinated to give rise to R_3 seeds. Table 4 summarizes the LT-B levels in R_1 seeds (one ear per event) and corresponding R_2 seeds (one to two ears per event). The LT-B levels in the R_2 seeds were greatly increased for all events (up to 100-fold in Event P77-9), compared with the LT-B levels in R_1 seeds. While Event P77-7 had a moderate LT-B level in R_1 seed (0.029%), it reached over 2% of TAEP in two ears tested in R_2 seeds. The R_2 plants of this event were grown in the field in the summer of 2001 for R_3 seeds. Results of LT-B levels from eight R_3 ears representing Event P77-7 are presented in Table 5. Significant differences ($p>0.0001$), were observed between ears descending from the same R_2 plant, and like P51 R_3 kernels, large standard deviations indicate differences in expression from kernels on the same ear. The highest reached 3.7% in ear #1. Based on the mass of the powder used and the volume of buffer added, the LT-B level in the ear could be expressed as 350 μg/g or 35 kg/ton LT-B of dry ground kernel.

Unlike P51 plants, in which the R_3 plants produced several-fold more LT-B than the R_2 generation, differences in LT-B gene expression between the R_2 and R_3 generations of event P77-7 were not very large. Levels of 2-3% observed in the R_2 generation are within the range of expression of most of the P77-7 plants analyzed in the R_3 generation, where levels of expression varied from 0.5 to 3.7% LT-B in TEAP.

Discussion

We describe the generation and characterization of LT-B expressing maize plants transformed with four different constructs. Several factors can be manipulated to control the level of expression of foreign genes in transgenic plants. These include foreign gene

sequence optimization, promoters, terminator sequences, and targeting and retention sequences. We examined the role of two of these factors, promoters and the microsomal retention signal SEKDEL, in expression of the synthetic LT-B gene and accumulation of the functional product in callus and seed.

For all constructs, transformation was confirmed by PCR analysis of herbicide-resistant callus clones, followed by genomic Southern blot hybridization analysis of DNA from the regenerated plants or their progeny. The rate of co-transformation of *bar* and LT-B genes ranged from 58 to 89% as determined by PCR. Results of Southern blot analysis show that most transgenic events had multiple copies of the transgene cassette, ranging from two to more than 10 copies in most cases. While we could not conclusively associate the level of LT-B expression with transgene copy number within individual constructs, high LT-B expression was generally associated with relatively low gene copy number. Event P51-62 was estimated to have about three copies of the gene, event P77-7 had about five copies and event P112-51 had about three copies. All these events had substantially higher LT-B expression relative to other events transformed with the same construct. Northern analysis was undertaken for the P51 events to determine the size of the transcription unit. An ~700-nucleotide transcript was observed, a size consistent with a transcript containing part of the TEV leader, the LT-B gene, and part of the terminator sequence. In most cases, plants that were confirmed to be transgenic by Southern blotting were also shown to express the 700-nucleotide gene transcript in Northern blot analysis and to express the protein in ELISA analysis.

The codon bias of a transgene is important in enhanced expression of a foreign protein (Gribskov et al., 1984). Mason et al. (1998) synthesized the LT-B gene used in this

study with a codon bias optimized for expression in potato and maize and demonstrated that the synthetic gene could be expressed in potato. The construct pTH210 (P51), with the constitutive CaMV 35S promoter, was used to determine if the codon usage in the synthetic LT-B gene had been adequately optimized for expression in maize. Expression of LT-B in callus of P51 and P65 events indicated that the synthetic LT-B gene was adequately optimized for expression in maize. LT-B levels in both P51 and P65 plants varied considerably between events, and did not exceed 0.04% of aqueous extractable protein.

We observed that transgenic callus events with high levels of LT-B protein in P51 but not P65 were associated with poor plant growth and fertility. Reduced performance in plant growth, vigor, and seed set in P51 plants relative to plants carrying other constructs could be attributed to several possible causes. Poor plant performance in some events could have been induced by *in vitro* tissue culture stress. Alternatively, it could be attributed to possible toxic effects of the LT-B protein in the tissues of the growing plant and tissues involved in seed development. We did not examine enough high LT-B-expressing P51 and P65 events to distinguish between these possibilities. This phenomenon was also observed by Mason et al. (1998) in transgenic potato plants producing high levels of LT-B protein. One of their higher-expressing transgenic potato events had poor growth and stunting, leading to the speculation that LT-B could in fact be toxic if expressed in the growing plant cells. P65 events did not have enhanced expression but had improved plant vigor compared with P51 plants. Retention of the LT-B protein in the ER could have limited its secretion into the cell wall and thus have reduced the effect of the protein on cell growth. It was concluded that low LT-B expression levels with the CaMV 35S promoter warranted investigation of LT-B expression using a seed-specific promoter.

Zeins constitute 50-60% of the seed protein in maize (Marks et al., 1985), and the 27 kDa gamma zein promoter is one of the strongest seed-specific promoters characterized to date. Substantially higher levels of LT-B expression were observed in the R_1 kernels of P77, up to 0.07% LT-B in TAEP, compared with 0.01% in P51 R_1 kernels. In the R_3 generation, the gamma zein promoter resulted in 13-fold higher levels (0.28% in P51-62 compared with 3.7% in P77-7) of LT-B in the seed than the constitutive CaMV 35S promoter. In P77 plants, LT-B protein accumulation was observed predominantly in the endosperm. This was determined through embryo vs. endosperm extraction and quantitation of LT-B (data not shown). Events from P112 had the highest level of expression in the R_1 seed (Figure 5). Since this construct was identical to P77 except for the SEKDEL motif included in the LT-B gene in construct pRC4-1, this increase in LT-B level could be attributed to the presence of this endoplasmic reticulum-retention sequence in the transgenic plants carrying pRC4-1. The SEKDEL motif is expected to sequester the protein in the ER (Munro and Pelham, 1987). Haq et al. (1995) showed that the SEKDEL motif resulted in significantly higher LT-B accumulation when compared with the LT-B gene without SEKDEL. These authors proposed that the cellular compartmentation of the SEKDEL protein could have facilitated oligomerization of LT-B monomers into pentamers detectable by ganglioside-dependent ELISA. We observed no marked increase in the level of LT-B protein in maize callus of P65 events in which the LT-B gene had the SEKDEL motif compared with P51 callus events, which did not. Why the enhancement effect of the SEKDEL motif was not observed in callus is not clear. It is possible that callus is a tissue that inherently does not contain as much protein as seed, and so does not accumulate protein beyond a certain level. No substantial SEKDEL enhanced expression was observed in kernels using the constitutive

promoter either; the highest level of expression observed in P65 R_1 kernels was 0.01% in event P65-69.

There appears to be an interaction between promoter and/or target tissue and the SEKDEL motif. More likely there is an interaction between the tissue and the SEKDEL motif; SEKDEL works in some tissues better than others due to differences in endomembrane system functions. To propose promoter SEKDEL interaction would imply interaction at the DNA level, which could be possible but not likely. These observations suggest the possible benefits of using the SEKDEL motif in improving the expression of a novel protein in maize kernels could be dependent on target tissue and promoter used, among other factors.

In all constructs, variability in LT-B levels was observed in plants from different transgenic events in all generations studied, and also between plants within the same event. Variability between events within the same generation could be explained as differences in transgene copy number and position effects in independently transformed events. Plants representing different transgenic events for each construct were analyzed for the presence of the LT-B gene by Southern blot analysis. The LT-B gene copy number in most cases was present in multiple copies for all constructs, ranging from two to greater than ten. Plants from the same transgenic event appeared to have the same band pattern as expected. However, despite having similar genetic makeup, significant differences in LT-B expression were sometimes observed between plants from the same event, especially in the R_3 generation (Table 5). Large standard deviation values were observed for kernels within the same ear. While some of this deviation could be attributed to experimental error, measurement of sample values in duplicate for each kernel was undertaken to reduce the

measurement error and so most of this variation is not due to experimental. Hood et al. (1997) observed variation of avidin expression ranging from 2.1% to 5.7% of aqueous-extractable protein between plants within the same transgenic event. These observed variations between individual plants in the same event are less readily explained. Some variation could be attributed to the microenvironment in which the plants developed, and some could be attributed to the genotypes of the plants. While the plants should be similar in genetic makeup, the Hi-II germplasm used for transformation was generated from crossing Hi-II parents A and B, which are derived from the inbred lines A188 and B73. The germplasm is therefore to a certain extent heterozygous and segregating to give some of the observed variation (Armstrong at al., 1991; M. Lee, personal communication). Some of the variation could be attributed to epigenetic effects. Other researchers have reported similar variation and proceeded to select the highest-expressing plant for subsequent generations (Zhong et al., 1999). These observations have important implications for commercial production of a novel protein. It cannot be assumed that plants from the same event have similar levels of expression, and it may take several generations of selection of individual lines to establish a stable transgenic line with a desired and consistent level of transgene expression.

Substantial increases in LT-B protein were observed between subsequent generations for both P51 and P77 events. In one case an increase of more than a 100-fold was achieved (Event P77-9, Table 4). It is possible that a foreign protein is poorly expressed in the R_1 generation because of the stress of the *in vitro* culture, transformation, and regeneration processes. Self-pollination in subsequent generations may increase the dose of the transgene in the endosperm, which might result in increased LT-B levels. Other workers have observed

that subsequent generations have been shown to have increasing novel protein levels relative to the R_1 generation (Zhong et al., 1999).

In the expression of an antigen in maize, the issue of dosage requirement is critical. Using the gamma zein promoter, LT-B levels of up to 350 μg/g of dry kernel tissue were attained. This is more than was required to induce a protective immune response in experimental mice. Mason et al. (1998) suggested that up to 1.1 mg would be required to induce a protective immune response in humans, and this dosage requirement could be met in 3g grams of dry maize meal. We harvested three generations of functional LT-B-expressing plants; the gene was not silenced in these generations and transmission was in a normal Mendelian fashion. Assays of LT-B in greenhouse and field showed an increase in expression of LT-B over the generations. Stability of expression is critical for high-expressing events that would be used for protein production in a practical situation. Variation in protein expression would have important implications on the amount of maize required for a specific dosage under direct administration or for product purification. In this study, the observed variation in LT-B levels could be attributed to environmental effects, segregation of the Hi-II background, epigenetic regulation of the transgene, or a combined effect of all these factors. Our observations have important implications on how to select the transgenic event with the appropriate level of gene expression for practical production of the candidate protein. The gamma zein promoter is certainly better than the CaMV 35S the promoter of choice for seed expression of LT-B, directing gene expression in the target organ at levels required for dosage. Other modifications such as the SEKDEL motif show a marked improvement in expression of this gene when combined with the gamma zein promoter. Out-crossing the transgenic plants to an elite line with good agronomic qualities

could potentially enhance the absolute amount of LT-B produced even further by increasing general agronomic performance.

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References

Armstrong CL, Green CE, Phillips RL: Development and availability of germplasm with high type II culture formation response. Maize Genet Coop Newsl 65: 92-93 (1991).

Austin S, Bingham ET, Koegel RG, Matthews DE, Shahan MN, Straub RJ, Burgess RR: An overview of a feasibility study for the production of industrial enzymes in transgenic alfalfa. Ann NY Acad Sci 721: 234-244 (1994).

Bradford MM: A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72: 248-254 (1976).

Chikwamba RK, Cunnick J, Hathaway D, McMurray J, Mason H, Wang K: A functional antigen in a practical crop: LT-B producing maize protects mice against *Escherichia coli* heat labile enterotoxin (LT) and Cholera toxin (CT). Transgenic Res. In press (2002).

Christou P: Transformation technology. Trends Plant Sci 1: 423-431 (1996).

Frame BR, Zhang H, Cocciolone SM, Sidorenko LV, Dietrich CR, Pegg SE, Zhen S, Schnable PS, Wang K: Production of transgenic maize from bombarded type II callus: Effect of gold particle size and callus morphology on transformation efficiency. In Vitro Cell Dev Biol-Plant 36: 21-29 (2000).

Gallie DR, Tanguay RL, Leathers V: The tobacco etch viral 5' leader and poly(A) tail are functionally synergistic regulators of translation. Gene 165: 233-238 (1995).

Gribskov M, Devereux J, Burgess RR: The codon preference plot: Graphic analysis of protein coding sequences and prediction of gene expression. Nucl Acids Res 12: 539-549 (1984).

Haq TA, Mason HS, Clements JD, Arntzen CJ: Oral immunization with a recombinant bacterial antigen produced in transgenic plants. Science 268: 714-716 (1995).

Hood EE, Witcher DR, Maddock S, Meyer T, Baszczynski C, Bailey M, Register J, Marshall L, Bond D, Kulisek E, Kusnadi A, Evangelista R, Nokolov Z, Wooge C, Mehogh RJ, Hernan R, Kappel WK, Ritland D, Li CP, Howard JA: Commercial production of avidin from transgenic maize: Characterization of transformant, production, processing, extraction and purification. Mol Breed 3: 291-300 (1997).

Krebbers E, Bosch D, Vandekekerekhove J: Prospects and progress in the production of foreign proteins and peptides in plants. In: Shewry PR, Gutteridge S (eds) Plant Protein Engineering, pp 315-325. Cambridge University Press, Cambridge, UK (1993).

Marks MD, Lindell JS, Larkins BA: Quantitative analysis of the accumulation of zein mRNA during endosperm development. J Biol Chem 260: 16445-16450 (1985).

Mason HS, Haq TA, Clements JD, Arntzen CJ: Edible vaccine protects mice against *Escherichia coli* heat labile enterotoxin (LT): Potatoes expressing a synthetic LT-B gene. Vaccine 16: 1336-1343 (1998).

Millar DG, Hirst T, Snider DP: *Escherichia coli* heat labile enterotoxin B sub-unit is a more potent mucosal adjuvant than its closely related homologue, the B sub-unit of Cholera toxin. Infect Immun 69(5): 3476-3482 (2001).

Munro S, Pelham HRB: A C-terminal signal prevents secretion of luminal ER proteins. Cell 48: 899-907 (1987).

Murray MG, Thompson WF: Rapid isolation of high-molecular-weight plant DNA. Nucl Acids Res 8: 4321-4325 (1980).

Pen J, Sijmons PC, Van Ooijen AJJ, Hockema A: Protein production in transgenic crops: Analysis of plant molecular farming. In: Industrial Crops Production. pp. 241-250. Elsevier, Amsterdam (1993).

Rhee Y, Staswick PE: Nucleotide sequence for the soybean vegetative storage protein vspB gene. Plant Physiol 98:794-5 (1992).

Sambrook J, Frittsch EF, Maniatis TH (eds): Molecular Cloning, A Laboratory Manual (2nd ed) Chapters 9-31 to 9-62, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY (1989).

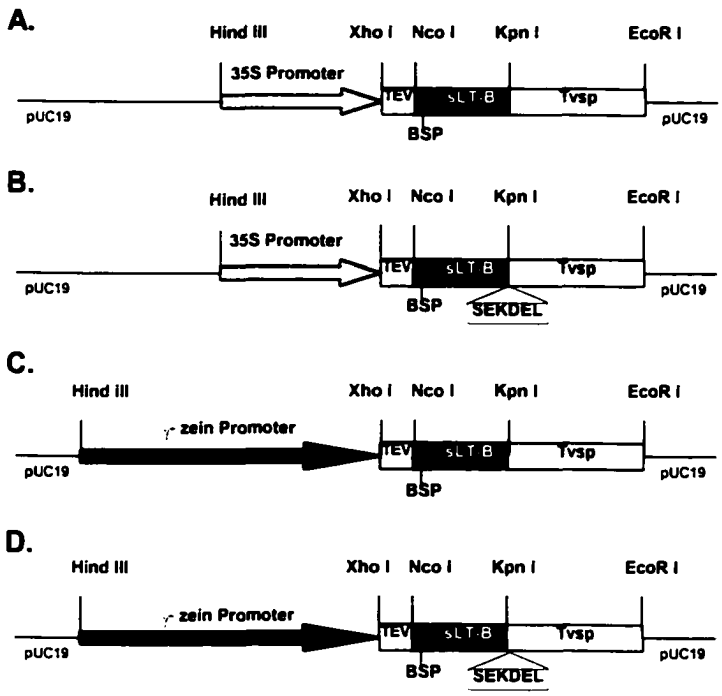
Sangtong V, Mottl EC, Long MJ, Lee M, Scott MP: Serial extraction of endosperm drillings (SEED)-A method for detecting transgenes and proteins in single viable maize kernels. (*Protocols*) Plant Mol Biol Rep 19: 151-158 (2001).

Spangler, BD: Structure of Cholera toxin and the related *Escherichia coli* heat-labile enterotoxin. Microbiol Rev 56:622-647 (1992).

Streatfield SJ, Jilka JM, Hood EE, Turner DD, Bailey MR, Mayor JM, Woodard SL, Beifuss KK, Horn ME, Delaney DE, Tizard IR, Howard JA: Plant based vaccines: Unique advantages. Vaccine 19: 2742-2748 (2001).

Tacket CO, Mason HS, Lasonsky G, Clements JD, Levine MM, Arntzen CJ: Immunogenicity in humans of a bacterial antigen delivered in a transgenic potato. Nature Med 4: 607-609 (1998).

Zhong GY, Peterson D, Bailey M, Witcher DR, Register J, Bond D, Li CP, Marshall L, Kulisek E, Ritland D, Meyer T, Hood EE, Howard JA: Commercial production of aprotinin in transgenic maize seed. Mol Breed 5: 345-356 (1999).



Construct	Transgenic maize event ID	LT-B expression in
pTH210	P51	Callus and whole plant
pTHK210	P65	Callus and whole plant
pRC4	P77	Seed
pRC4-1	P112	Seed

Figure 1. Schematic diagram of constructs used for maize transformation to generate LT-B-expressing transgenic plants. All constructs contain the synthetic gene encoding the B subunit of LT in pUC19 vector background. 35S Promoter, CaMV 35S promoter; γ-zein promoter, 27 kDa gamma zein promoter from maize; TEV, tobacco etch virus translational enhancer leader sequence; sLT-B, the synthetic LT-B gene; Tvsp, soybean vegetative storage terminator; SEKDEL, endoplasmic reticulum-retention sequence motif; BSP, bacterial signal peptide from LT.

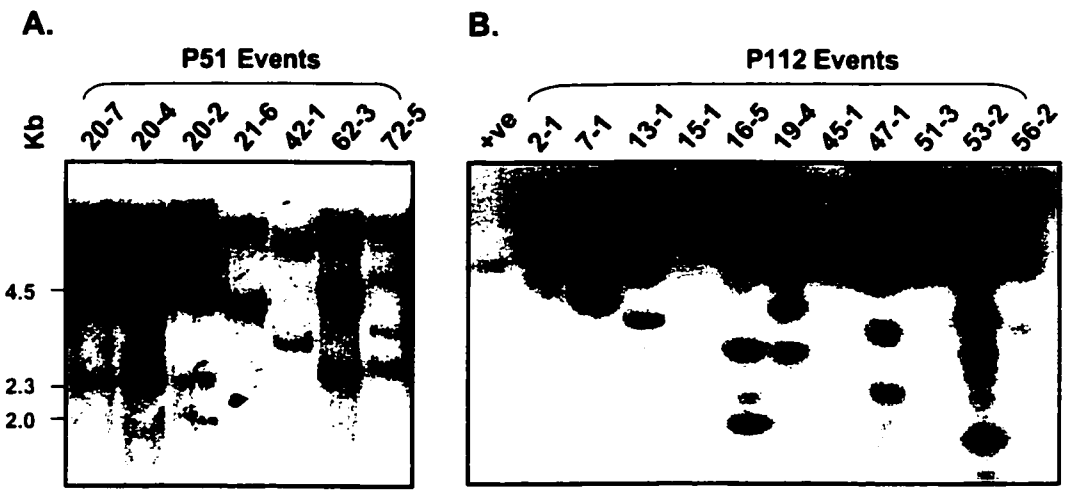


Figure 2. Southern blot of total leaf genomic DNA of P51 (A) and P112 (B) events digested with *Hind* III and *Nco* I respectively and probed with the LT-B gene.

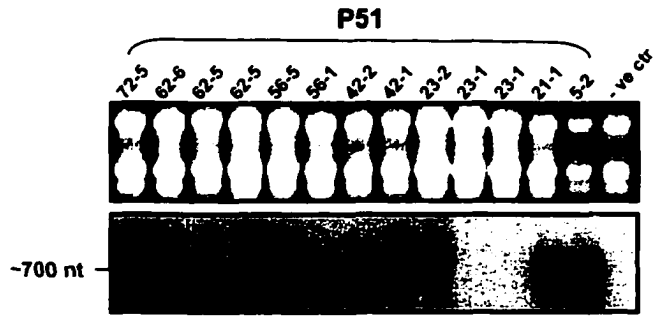


Figure 3. Northern blot (A) analysis of total leaf RNA of P51 (B) probed with a ^{32}P-labeled LT-B gene fragment RNA from non-transgenic B73 plant used as negative control.

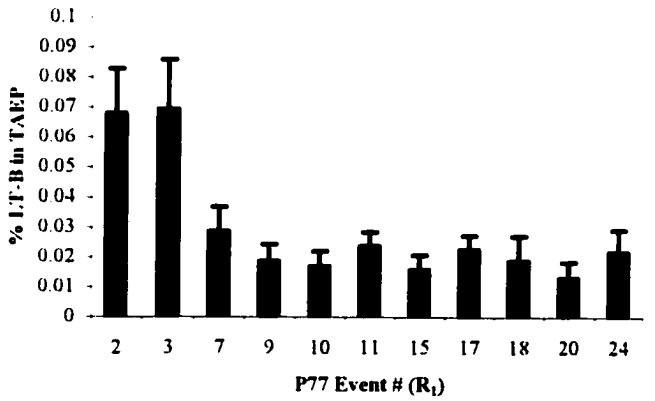


Figure 4. LT-B level (% in total aqueous extractable protein) in R1 seeds of P77 events. Error bars represent ± standard deviation.

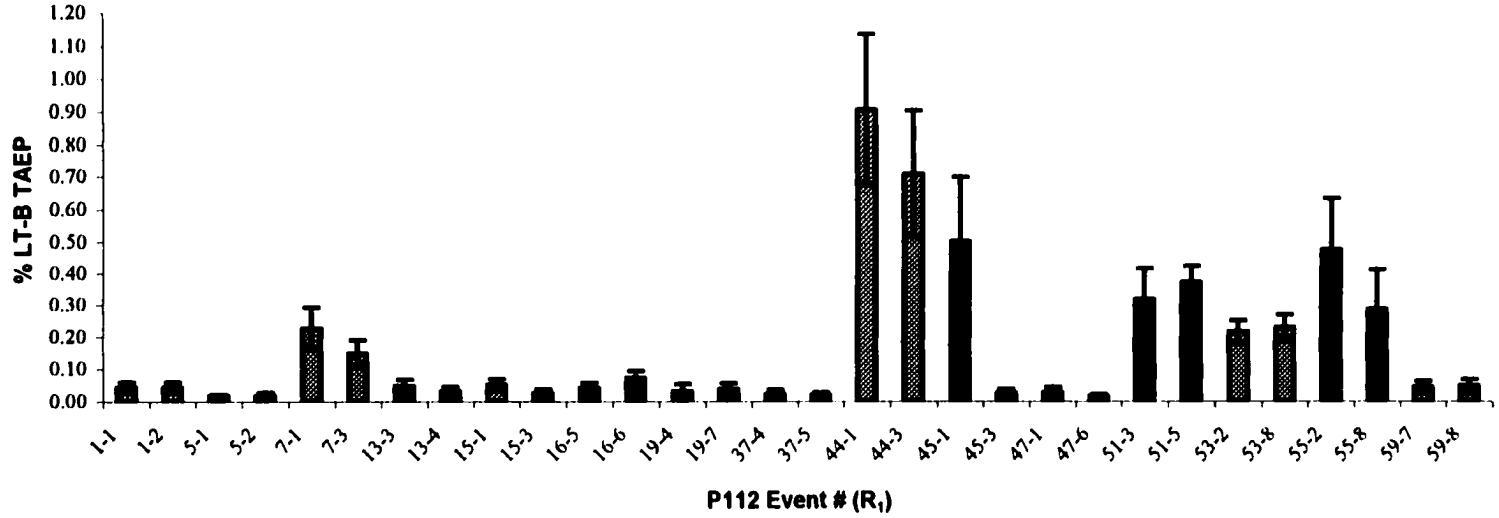


Figure 5. LT-B [% in total aqueous extractable protein TAEP in R1 seeds of P112 events. Data from ears in the same event are shaded similarly. Errors bars indicate ± standard deviation.

Transgenic plant ID	Total # events tested	# Events expressing LT-B at various levels*		
		<0.005	0.005-0.01	0.01-0.04
P51	54	42	9	3
P65	51	33	13	5

* Level of assembled LT-B protein in total aqueous extractable protein was determined by ganglioside-dependent ELISA.

Table 1. Level of assembled LT-B protein (% LT-B in total aqueous-extractable protein) in transgenic maize callus.

Transgenic plant ID	Total # events tested	# Events expressing LT-B at various levels*			
		<0.005	0.005-0.01	0.01-0.1	> 0.1
P77	19	2	6	11	0
P112	15	0	0	9	6

* Level of assembled LT-B protein in total aqueous extractable protein was determined by ganglioside-dependent ELISA.

Table 2. Level of assembled LT-B protein (% LT-B in total aqueous-extractable protein) in R1 transgenic seeds.

Transgenic plant ID	Total # events tested	# of events with seed set*				% events set seed >50 kernels
		0	<50	50-100	101-200	
P51	13	5	5	1	2	23
P65	14	0	5	7	2	64
P77	19	0	5	5	9	74
P112	20	1	8	8	3	55

Table 3. Summary of seed set from transgenic maize carrying four different constructs

	R_1 seed		R_2 seed			Fold increase (R_2/R_1)
Event ID	Mean	SD	Ear ID	Mean	SD	
2	0.068	0.015	2-1	1.155	0.342	18.5
			2-2	1.356	0.373	
3	0.069	0.017	3-1	1.319	0.627	9.1
			3-2			
7	0.029	0.008	7-1	2.071	0.816	84.5
			7-2	2.761	0.817	
9	0.019	0.006	9-1	1.634	0.358	104.7
			9-2	2.246	1.077	
10	0.017	0.005	10-1	0.324	0.141	19.3
			10-2	0.327	0.140	
11	0.024	0.005	11-1	0.463	0.246	14.3
			11-2	0.211	0.100	
17	0.023	0.005	17-1	0.127	0.057	5.6
18	0.019	0.008	18-1	0.303	0.222	16.9
			18-2	0.292	0.117	

Table 4. Levels of assembled LT-B protein (% LT-B in total aqueous-extractable protein) in R_1 and R_2 seeds of P77.

Event ID	Ear ID	Mean	SD	t Grouping*
P51-62	1	0.282	0.168	a
	2	0.252	0.199	a
	3	0.183	0.111	ab
	4	0.167	0.039	b
	5	0.166	0.091	abc
	6	0.148	0.052	c
	7	0.121	0.086	cd
	8	0.026	0.019	d
P77-7	1	3.660	2.740	a
	2	2.096	1.334	b
	3	1.743	1.094	bc
	4	1.350	0.552	bc
	5	1.318	0.780	bc
	6	1.299	0.752	bcd
	7	0.949	0.659	cd
	8	0.190	0.123	d

* values with same letter(s) were not significantly different from each other ($p \geq 0.05$).

Table 5. Comparison of LT-B levels (% LT-B in total aqueous extractable protein) in R3 seed of P51 and P77.

CHAPTER 3

Localization of a bacterial antigen in starch granules of transgenic maize kernels

Rachel Chikwamba, Paul Scott, Hugh Mason, Kan Wang (Authorship Tentative)

Manuscript in preparation.

Abstract

The B subunit of *Escherichia coli* heat labile enterotoxin (LT-B) is a potent oral immunogen, with potential for use as a vaccine and an adjuvant to other co-administered vaccine antigens. LT-B expressed in plants was shown to be functional and immunogenic in animals and humans. Using immunogold labeling/electronmicroscopy and western analysis, we show here that the LT-B proteins with the native bacterial signal peptide (BSP), accumulate in starch granules of transgenic maize kernels. No LT-B accumulation in starch granules was observed when a signal peptide from maize 27 kDa γ-zein protein was used to replace BSP in LT-B production. This represents the first case in which a bacterial protein with a native signal peptide is transported into the starch granules of a higher plant.

Introduction

The *Escherichia coli* heat labile enterotoxin B subunit (LT-B), a potent oral immunogen, has been used as a model antigen to demonstrate the feasibility of producing an effective oral vaccine in transgenic plants (Mason et al., 1998, Streatfield et al., 2001,

Chikwamba et al., 2002). Orally administered LT-B has been shown to elicit strong mucosal and serum antibody responses. When administered together with other antigens, LT-B stimulates strong immune responses to the co-fed proteins. LT-B's receptor binding capacity in the gut makes it an ideal adjuvant and carrier molecule for the delivery of other antigenic epitopes to the gut's mucosal system (Spangler 1992).

The likelihood of protein degradation in the stomach and uncertainty about the effectiveness of orally delivered antigens have raised concerns about the oral vaccination approach. The delivery of an oral vaccine in transgenic plant tissue could protect it from the low pH and digestive enzymes of the stomach as well as and prolong the antigen's exposure to the gut's immune system. The sub-cellular location of any novel protein expressed in plants is also important for their accumulation, folding and assembly, and, depending on their intended use, may have an effect on their functionality (Hood et al, 1997). Proteins are commonly targeted to extracytosolic compartments in both eukaryotic and prokaryotic cells, and this occurs by a variety of mechanisms. Translocation mechanisms usually involve the synthesis of a protein with a specific targeting signal, and the recognition of this signal by the appropriate translocation machinery (Walter, 1994). Numerous bacterial and viral proteins have been produced in transgenic plants. Some proteins could be transported into chloroplasts when they were fused to a chloroplast-targeting domain. However, little is known about the sub-cellular destinations for those prokaryotic proteins produced in transgenic plants with their native signal peptides. In this study, we examined the sub-cellular localization of the LT-B protein in transgenic maize kernels.

Materials and Methods

Constructs for maize transformation

A synthetic LT-B coding sequence (sLT-B) with codon usage optimized for both potato and maize expression was fused to DNA sequences encoding either the native LT-B signal peptide (BSP; Mason et al., 1998) or maize 27 kDa gamma zein signal peptide (ZSP; Marks et al., 1985), as described in Figure 1. The LT-B gene cassettes were regulated by the 27 kDa gamma zein promoter, an endosperm specific promoter.

Maize transformation and plant regeneration

Transgenic maize plants carrying constructs P77 and P81 were generated using the biolistic gun method (Frame et al., 2000) and transgenic plants were grown to maturity in the greenhouse. Over twenty independent transformation events from each construct were evaluated for the expression of the LT-B gene in kernel.

Evaluation of LT-B expression

Evaluation of LT-B expression was performed in transgenic maize kernels using ganglioside dependent enzyme linked immunosorbent assay as described by Chikwamba et al (2002).

Fixation of tissue for immunolocalization

Immature (23 days post-pollination) and mature (dried) maize kernels were sectioned in fixative (0.1M carcodylate buffer, 0.5% gluteraldehyde and 2% paraformaldehyde). Tissue blocks were incubated in fixative for 2 hours at 4°C and rinsed three times in 0.1M carcodylate buffer; 15 minutes each wash, on a rotating shaker. This was followed by a succession of dehydrating treatments as follows; tissue blocks were rinsed with 50% ethanol

for 15 minutes on a rotating shaker, followed by incubation with 70% ethanol for 2 hours at room temperature. The 70% ethanol was removed and replaced with 95% ethanol for a two-hour incubation, followed by three two-hour incubations with 100% ethanol. Tissue blocks were then incubated in a gradually increasing white London Resin (LR white), starting with 1:3, 2:1, 3:1 (V:V) LR white to ethanol for 8 – 12 hours each time and finally with 100% LR white overnight. Incubation in pure LR white was repeated twice for 8-12 hours each time after which the tissue blocks were cast for in gelatin capsules for 24-48 hours at 60°C. Ultra thin sections were cut and mounted on grids for scanning transmission electron microscope (STEM) by the Bessey Microscopy Facility at the Iowa State University.

Immunogold labeling

To detect sub-cellular localization of LT-B expression in transgenic maize kernels, tissues were analyzed by immunogold labeling at electron microscopic level following fixation of kernel tissue. Kernel sections mounted on grids were incubated in TBS blocking buffer [0.05M Tris, pH8.3, 0.85% NaCl, supplemented with 0.5% BSA, 0.5% normal donkey serum and 3% dry milk] for 2 hours at room temperature. This was followed by incubation of grids in goat anti-LT-B polyclonal sera (KPL) diluted 1:100 (V:V) in blocking buffer for two hours at 37°C. Negative control grids were incubated in fresh blocking buffer. Grids were washed three times, 10 minutes each wash in TBS buffer supplemented with 0.5% normal donkey serum and 0.5% BSA (no milk), and incubated at room temperature in donkey anti-goat polyclonal serum conjugated to 18nm gold, diluted 1:20 (V:V) in TBS buffer supplemented with 0.5% normal donkey serum and 0.5% BSA, 0.1% fish gelatin. Grids were washed three times, 10 minutes each wash in distilled water and allowed to air dry.

Starch isolation

To isolate starch, dry kernels were soaked in glass vials containing 2 to 4 mls of 0.45% $Na_2S_2O_5$ for 3 days, and the pericarp and embryo were removed using a razor blade. Endosperms were placed into a 50ml conical tube with 10ml water and homogenized with polytron. The polytron was rinsed three times with water in between samples. Homogenate was filtered with the aid of a vacuum pump through plastic Millipore stopper apparatus (250ml) with a 30μM nylon filter cut to size (Spectrum cat#146506) in a side-arm flask, and the filtrate collected in a 50ml conical tube. Homogenates were allowed to settle at for at least one and a half hours at 4°C. Water was removed from the sample by aspiration, and the remaining starch water slurry transferred to a 2ml tube and the slurry was centrifuged at low speed for 5 minutes. Water was carefully removed and the starch washed three times with 1.5ml water. The starch was again washed three times with one of three reagents, 75% ethanol, 95% ethanol or 75% ethanol with 3% β-mercaptoethanol. Samples were dried completely in a speed-vac. To remove exogenous proteins, samples were incubated with 5mM $CaCl_2$ with 100μg/ml Thermolysin (Sigma) at 62°C for about 16 hours (overnight). Samples were then boiled in SDS sample buffer (1 ml per 50mg starch) for 10 minutes at 100°C for western analysis.

Western Blot analyses

Western blot analyses were performed with goat polyclonal serum against LT-B followed by goat anti-rabbit polyclonal serum conjugated to alkaline phosphatase as described (Chikwamba et al, 2002, Chapter 4).

Results and discussion

The maize-synthesized LT-B proteins produced by plants carrying the BSP/sLT-B construct (P77) were structurally and functionally identical to the bacterium-derived LT-B protein. Oral immunization with maize-synthesized LT-B induced a strong immune response in BALB/c mice, protecting them from challenge with the *E. coli* labile toxin (LT) and its homologue Cholera toxin (CT) (Chikwamba et al, 2002, Chapter 4 of dissertation).

Immunogold labeling was used to detect the sub-cellular localization of LT-B within transgenic maize kernels. Ultra thin sections of 23-day old immature kernels from P77 transgenic maize plants were processed as described and viewed under the transmission electron microscope. As shown in Figure 2B, LT-B protein was detected exclusively in the starch granules within the amyloplasts of immature kernels of P77. No gold particles could be detected in the non-transgenic maize immature kernel control (Figure 2C). In P77 kernels, gold particles were not detected near or within cell walls, intercellular spaces, in the endoplasmic reticulum (ER), Golgi apparatus or secretory vesicles.

To distinguish whether the LT-B proteins were localized at the surface or inside of the starch granules, starch samples from mature transgenic seeds were collected, treated to remove surface proteins and examined using western analysis. The approach was based on the fact that polypeptides that are within the starch granules are not susceptible to hydrolysis upon treatment of intact granules with exogenous proteases (Mu-Forster and Wasserman, 1998). Starch samples were isolated from individual mature kernels of transgenic maize P77, using three different methods (75% ethanol, 95% ethanol or 75% ethanol plus 3% β-mercaptoethanol) to remove external proteins. The starch samples were then treated with Thermolysin overnight to remove the remaining external proteins that were not embedded in

the starch granules. The samples were washed 5 times in 1.5 ml distilled water to remove the Thermolysin. The samples were boiled to release any proteins that are embedded in starch. Duplicated gels containing equal amounts of protein sample were analyzed by western blotting using antibodies against either LT-B or zein proteins. The zein proteins are the major seed storage protein in maize, comprising 50-60% of endosperm protein (Marks et al. 1985) and are localized outside of the starch granules (Mu-Forster and Wasserman, 1998). We used the zein antibody as a control to indicate the presence or absence of the external proteins of starch samples. As shown in Figure 3A and B, both LT-B and zein proteins could be detected in the starch preparation treatments using 75% or 95% ethanol (Lanes 2 and 3). However, only LT-B protein could be detected when the starch samples were prepared from the treatment of 75% ethanol plus 3% B-mercaptoethanol (Lanes 1). LT-B was not detected in non-transgenic samples following any of the treatments (Lane 4 in Figure 3A&B). This result strongly indicated that the LT-B proteins were present within the starch granules. This further confirmed the observation made with immunogold localization that LT-B proteins localized in starch granules of immature kernels.

To verify whether the starch localization of LT-B protein required the bacterial signal peptide, starch samples of transgenic maize kernels (P81) carrying construct ZSP/sLT-B cassette (Figure 1B) were prepared as for P77. In contrast to P77 starch sample, P81 starch sample treated with the 75% ethanol plus 3% B-mercaptoethanol showed no accumulation of LT-B proteins inside of the starch granules (Lanes 3 vs 4, Figure 3C), while the LT-B could be detected in P81 seed (Lane 2, Figure 3C). This result indicated that the native signal peptide of LT-B is necessary for the starch localization of the LT-B protein in transgenic maize kernel.

We have demonstrated here the localization of a bacterial protein with its native signal peptide in the starch granules of transgenic maize seed. This observation was contrary to our expectation, in which LT-B protein in transgenic maize kernels would be ER targeted and ultimately secreted to the extracellular spaces (Ellgaard et al, 1999). In *E. coli*, LT-B is targeted to the periplasmic space (Hirst et al., 1995). LT-B expressed in yeast was shown to be ER targeted, assembled into pentamers and retained within the endomembrane system without secretion (Schonberger et al., 1991). Studies in *E. coli* showed that enzyme-catalyzed disulfide bond formation was necessary for LT-B assembly (Hirst et al., 1995), and in eukaryotic cells this process is facilitated by protein disulfide isomerase (PDI) in the ER. The ER is the site of synthesis and maturation of proteins destined for secretion, for the plasma membrane, and for the secretory and endocytic organelles. Mason et al, (1998) expressed LT-B in potato tubers and speculated it to be targeted to the ER where the essential enzymes that catalyze folding reside. In plants, the default pathway for proteins that are transported through the ER is secretion, although a protein's ultimate sub-cellular localization may also depend on factors other than just the presence of signal sequence, including topological information on the protein itself (Stachelin and Moore, 1995).

Molecular weight analysis in SDS-PAGE (Chikwamba et al., 2002) of maize-synthesized LT-B protein indicated that the peptide had an apparent mass 11.6 kDa, which is consistent with the expected mass of LT-B from which the signal peptide was removed. The combination of this with the observation that no LT-B accumulation in starch was detected when the bacterial signal peptide was replaced with the zein signal peptide described in this study provide evidence that the 21 amino acid LT-B signal peptide is responsible for the targeting of LT-B into the amyloplast, where it is then integrated into the starch granules.

Computer analysis on the signal peptide (SignalP V1.1 World Wide Web Server) did not reveal any obvious sequence characteristic of plastid targeting peptides.

The mechanism for the plant starch granule targeting by a signal peptide bacterial protein described in this study is not clear. It is possible that the bacterial signal peptide can target the conjugated protein into a plant plastid, due to its prokaryotic origin (Shih et al., 1986). Most plastid proteins are nuclear encoded, synthesized as large precursors in the cytosol and post-translationally imported into the organelle, and into the appropriate compartments across outer and inner membranes. Protein entry into the stroma plastids has been termed the general import pathway and is directed by the stroma-targeting domains in transit peptides (Cline and Henry, 1996). The majority of precursor proteins have a cleavable transit peptide, which includes a stromal targeting domain that initiates transport of the precursor through the general import pathway (von Heijne et al, 1989). Using the barley aleurone α-amylase signal peptide, Düring et al (1990) successfully targeted immunoglobulin chains to intracellular spaces in transgenic tobacco. This group observed localization of the assembled monoclonal antibodies in the endoplasmic reticulum and unexpectedly, in the chloroplasts. No particular features of the immunoglobulins they were working with were expected to target the chloroplasts, and therefore they could not explain the specific localization, which they observed. The fact that the antibodies were assembled in chloroplasts suggests that there may be folding machinery in the organelles, which can handle external proteins.

It is also possible a retro-translocation mechanism is involved, where by proteins are exported from the lumen or membrane of the ER into the cytosol (Tsai et al, 2002). However, in such a case, the LT-B proteins would be targeted to the ER with the amyloplast

as the final destination. This process would require a mechanism for the relocation of the LT-B out of the ER into the cytoplasm, and from the cytoplasm into the amyloplast, where the LT-B then becomes imbedded in starch as observed. Retro-translocation is poorly understood, and it is thought to be involved in the quality control of proteins, where misfolded proteins are returned to the cytosol and degraded (Tsai et al, 2002). Retro-translocation from the ER has been reported for the translocation of the A subunits of the Cholera and *E. coli* labile toxins (Lord and Roberts, 1998, Sandvig et al, 2000, Tsai et al, 2002). Whether the B subunits undergo a similar process was not determined. Analysis of LT-B (SOSUI, World Wide Web Server) shows two major hydrophobic domains other than the signal peptide. The presence of these hydrophobic domains on the LT-B protein, suggests that this method of translocation could be responsible for the exit of LT-B from the ER, if it was targeted there to begin with. In this scenario, the subsequent insertion into the amyloplast could occur as described for transportation of a protein without a cleavable signal peptide. At least one pathway has been elucidated for translocation of peptides without a cleavable signal into the amyloplast. Insertion of these proteins does not require hydrolysis of nucleotide triphosphates, nor does it appear to depend on the proteins of the plastid membranes. It is likely that the partitioning into the lipid bilayer of hydrophobic domains within these proteins controls their insertion into the membrane (Li and Chen, 1996).

Further investigation on the starch targeting property of the LT-B signal peptide using marker genes will contribute to the elucidation of mechanisms of protein translocation to plastids. In addition, the localization of LT-B protein in maize starch has important implications on the effectiveness of a maize-based vaccine. Animal studies (Chikwamba et al, 2002) showed that mice orally immunized with maize meal expressing LT-B induced a

much higher serum and mucosal response than non-transgenic maize meal mixed with an equivalent amount of bacterial LT-B. Encapsulation of the antigen in starch granules could result in protection from the harsh environment of the stomach and possibly in slow release from the starch and a consequent prolonged exposure of antigen to the gut associated mucosal system. The bacterial signal peptide could find utility in encapsulation of novel proteins in starch. By translationally fusing a novel protein to the LT-B signal peptide, the novel protein could be targeted within the starch grain, and because starch extraction from maize is a well established procedure, protein extraction would be greatly facilitated.

References

Cline, K and Henry, R, (1996). Import of nucleus encoded chloroplast proteins. *Annual Reviews of Cellular and Developmental Biology* **12**: 1-26.

Chikwamba RK, .Cunnick, J, Hathaway, D, McMurray, J, Mason, H, and Wang, K, (2002). A functional antigen in a practical crop: LT-B producing maize protects mice against *Escherichia coli* heat labile enterotoxin (LT) and Cholera toxin (CT). *Transgenic Research* **1122**: 1-15, In press.

Düring, K, Hippe, S, Kreuzaler, F and Schell, J, (1990). Synthesis and self assembly of a functional monoclonal antibody in transgenic *Nicotiana tabacum*. *Plant Molecular Biology* **15**: 281-293.

Ellgaard, I, Molinari, M and Helenius, A, (1999). Setting the standards: quality control in the secretory pathway. *Science* **286**: 1882-7.

Frame BR, Zhang, Z, Cocciolone, SM, Sidorenko, LV, Dietrich, CR, Pegg, SE, Zhen, S, Schnable, PS, and Wang, K, (2000). Production of transgenic maize from bombarded type II callus: Effect of gold particle size and callus morphology on transformation efficiency. *In Vitro Cellular Developmental Biology-Plant* **36**: 21-29.

Hartl, FU and Neupert, W, (1990). Protein sorting into the mitochondria: evolutionary conservations of folding and assembly. *Science* **247**: 930 – 938.

Hirst, TR, Hardy, SJS and Randal, LL, (1983). Assembly in vivo of enterotoxin from Escherichia coli: formation of the B subunit oligomer. *Journal of Bacteriology* **153**: 21-26.

Li, HM and Chen, LJ, (1996). Protein targeting and integration signal for the chloroplastic outer membrane. *Plant Cell* **8**: 2117-2126.

Lord, JM, and Roberts, LM, (1998). Toxin entry: retrograde transport through the secretory pathway. *Journal of Cell Biology* **140**: 733-736.

Mason HS, Haq, TA, Clements, JD and Arntzen, CJ, (1998). Edible vaccine protects mice against *Escherichia coli* heat labile enterotoxin (LT): Potatoes expressing a synthetic LT-B gene. *Vaccine* **16**: 1336-1343.

Marks, MD, Lindell, JS, Larkins, BA, (1985), Quantitative analysis of the accumulation of zein mRNA during endosperm development. *Journal of Biological Chemistry* **260**: 16445-16450.

Mu-Forster, C, and Wasserman, BP, (1998). Surface localization of zein storage proteins in starch granules from maize endosperm. *Plant Physiology* **116**: 1563-1571.

Shih, MC, Lazar, G, Goodman, HM (1986). Evidence in favor of the symbiotic origin of chloroplasts: primary structure and evolution of tobacco glyceraldehydes-3-phosphate dehydrogenases. *Cell* **47**: 73-80.

Schonberger, O, Hirst, TR, Pines, O, (1991). Targeting and assembly of an oligomeric bacterial enterotoxoid in the endoplasmic reticulum of Saccharomyces cerevicae. *Molecular Microbiology* **5**: 2663-2671.

Spangler, BD, (1992). Structure of Cholera toxin and the related *Escherichia coli* heat-labile enterotoxin. *Microbiological Reviews* **56**:622-647.

Sandvig, K and van Deurs, B, (2000). Entry of ricin and Shiga toxin into the cells: molecular mechanisms and medical perspectives. *The EMBO Journal* **19**: 5043-5950.

Stachelin, LA, and Moore, I, (1995). The plant Golgi apparatus: Structure, functional organization and trafficking mechanisms. *Annual Reviews Plant Physiology and Plant Molecular Biology* **46**: 261-288.

Streatfield SJ, Jilka JM, Hood EE, Turner DD, Bailey MR, Mayor JM, Woodard SL, Beifuss KK, Horn ME, Delaney DE, Tizard IR, Howard JA, (2001). Plant based vaccines: unique advantages. *Vaccine* **19**: 2742-2748.

Walter, P (1994) Signal sequence recognition and protein targeting to the endoplasmic membrane. *Annual reviews of cellular and developmental biology* **10**: 87-112.

Tsai, B., Ye, Y., and Rapaport, T.A., (2002). Retrotranslocation of proteins from the endoplasmic reticulum into the cytosol. *Nature* **3**: 246-255.

Von Heijne, G, Steppuhn, J, and Herman, RG, (1989). Domain structure of mitochondria and chloroplast targeting peptides. *Journal of Biochemistry* **180**: 535-545.

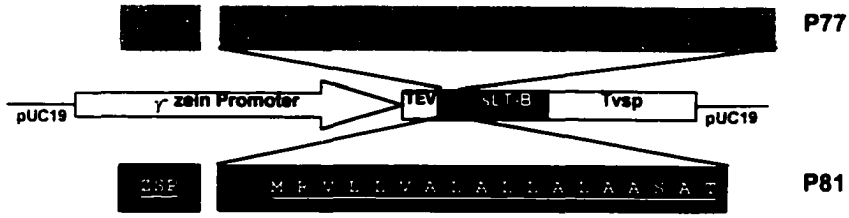


Figure 1. Schematic diagram of constructs used for maize callus transformation to generate LT-B-expressing transgenic plants. Both constructs are in a pUC19 vector and contain the maize 27 kDa; TEV, tobacco etch virus translational enhancer leader sequence; sLT-B, the synthetic LT-B gene; The Tvsp, soybean vegetative storage terminator; BSP, bacterial signal peptide from LT-B; ZSP, bacterial signal peptide from the maize 27kDa zein protein. Amino acids constituting each signal peptide are shown in their single letter form.

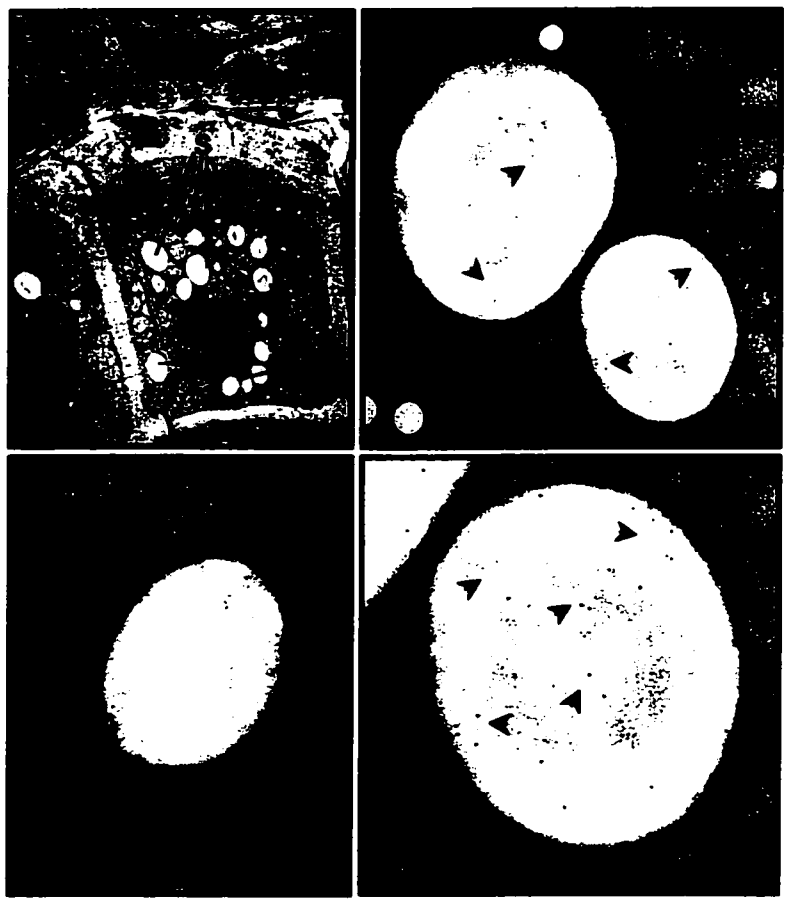


Figure 2. Immunolocalization of LT-B in immature maize kernels. (A) Section of maize kernel showing a cell beneath the aleurone layer, CM, cell membrane, N; nucleus, S; starch granules. (B) and (D), P77 transgenic kernel showing LT-B localization in starch grains, indicated by arrows. D is a close up of B. (C), Non-transgenic maize kernel as negative showing no gold labeling. .

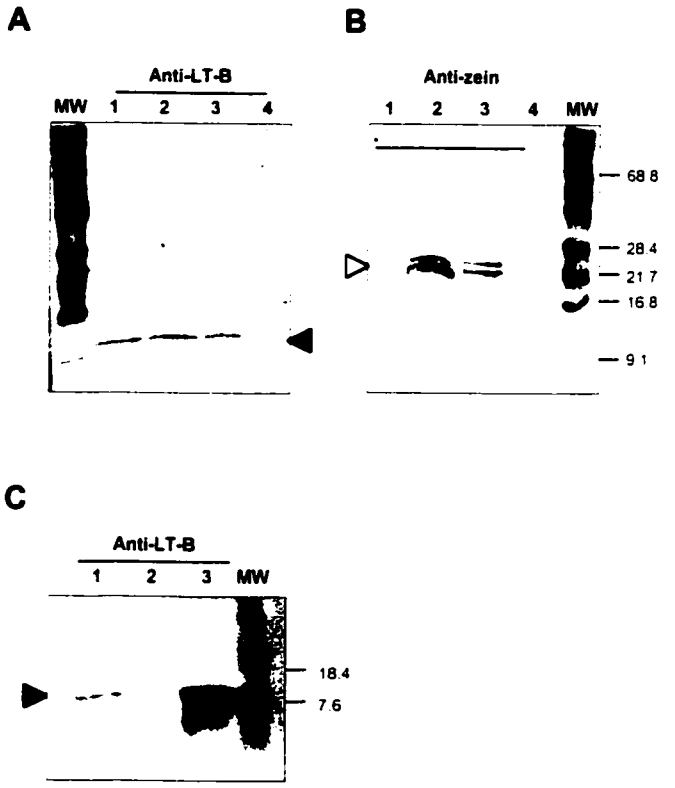


Figure 3. Western blot analyses of total proteins from starch samples treated to remove external proteins. Starch samples from mature maize seeds were treated with three different ethanol treatments prior to Thermolysin treatment as described in materials and methods. Samples were then boiled for 10 minutes at 100°C to release granule bound starch. Proteins were separated on a 12% SDS-PAGE and transferred to a 0.45 micron nitrocellulose membrane and probed with goat anti-LT-B antibodies (**A, C**) or rabbit anti-zein antibodies (**B**), followed by rabbit anti-goat and goat anti-rabbit alkaline phosphatase conjugate respectively. **(A and B)**, P77 transgenic starch treated with 75% ethanol plus 3% β-mercaptoethanol (Lane 1); 95% ethanol (Lane 2), 75% ethanol (Lane 3), B73 treated with 75% ethanol plus 3% β-mercaptoethanol (Lane 4), respectively. **(C)**, P77 transgenic kernel starch washed with 75% ethanol plus 3% β-mercaptoethanol (Lane 1), P81 starch sample washed with 75% ethanol plus 3% β-mercaptoethanol (Lane 2), P81 transgenic kernel total protein extracts (Lane 3).

CHAPTER 4

A functional antigen in a practical crop: LT-B producing maize protects mice against Escherichia coli heat labile enterotoxin (LT) and Cholera Toxin (CT)

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Abstract

We have produced a functional heat labile enterotoxin (LT-) B subunit of *Escherichia coli* in maize. LT-B is a multimeric protein that presents an ideal model for an edible vaccine, displaying stability in the gut and inducing mucosal and systemic immune responses. Transgenic maize was engineered to synthesize the LT-B polypeptides, which assembled into oligomeric structures with affinity for G_{M1} gangliosides. We orally immunized BALB/c mice by feeding transgenic maize meal expressing LT-B or nontransgenic maize meal spiked with bacterial LT-B. Both treatments stimulated elevated IgA and IgG antibodies against LT-B and the closely related cholera toxin B subunit (CT-B) in serum, and elevated IgA in fecal pellets. The transgenic maize induced a higher anti-LT-B and anti-CT-B mucosal and serum IgA response compared to the equivalent amount of

bacterial LT-B spiked into maize. Following challenge by oral administration of the diarrhea inducing toxins LT and CT, transgenic maize-fed mice displayed reduced fluid accumulation in the gut compared to non-immunized mice. Moreover, the gut to carcass ratio of immunized mice was not significantly different from the PBS (non-toxin) challenged control group. We concluded that maize synthesized LT-B had features of the native bacterial LT-B such as molecular weight, G_{M1} binding ability, and induction of serum and mucosal immunity. We have demonstrated that maize, a major food and feed ingredient, can be efficiently transformed to produce, accumulate and store a fully assembled and functional candidate vaccine antigen.

Abbreviations: *E. coli: Escherichia coli*; LT-B: labile toxin B subunit; PMSF: phenylmethanesulfonyl fluoride; PBS: phosphate buffered saline; SDS-PAGE: sodium dodecyl sulfate polyacrylamide gel electrophoresis; BSA: Bovine serum albumin; ELISA: enzyme linked immunosorbent assay; EDTA: ethylene-diamine tetraacetic acid; Tris: Tris hydroxymethyl aminomethane; G_{M1}: galactosyl-N-acetylgalactosamyl-sialyl-galactosylglucosyl ceramide.

Introduction

The *E. coli* heat labile enterotoxin B subunit protein (LT-B) has qualities of an ideal oral vaccine, showing stability upon ingestion resulting in stimulation of secretory IgA antibodies against specific virulence determinants of invading pathogens in the gut. Functional LT-B is synthesized as monomers, which assemble into pentameric structures with high affinity for G_{M1} (galactosyl-N-acetylgalactosamyl-sialyl-galactosylglucosyl

ceramide) gangliosides. It is a potent oral immunogen and has been demonstrated to elicit specific serum and mucosal antibodies (Spangler, 1992; Dickinson and Clements, 1996). In addition, the protein is relatively small and has immunological and other physiochemical properties that could be readily tested to determine its functionality. These features made it an ideal gene for plant expression and production.

The potential of crop plants for the production of biopharmaceuticals and other novel proteins is beginning to be realized. To date, efforts to produce these proteins in plants have focused on the dicotyledonary plants such as potato, tobacco, alfalfa and *Arabidopsis* (Moffat et al., 1995; Kusnadi et al., 1997; Mason et al., 1996; 1998; Tackaberry et al., 1999). These plants have well established and reliable transformation procedures via *Agrobacterium tumefaciens*-mediated transformation. Other attractive attributes of these plants include production of large volumes of green tissue in tobacco and alfalfa, which can produce several crops per year by cutting back foliage. Annual yields can be 25 and >100 metric tonnes (MT) per hectare for alfalfa and tobacco, respectively (Daniell et al., 2001). However, these plants have their drawbacks, especially for the purpose of convenient oral vaccine production and delivery. Green leaf tissues in which proteins are produced in these plants tend to have high amounts of phenolic and other potentially toxic compounds. Tobacco and *Arabidopsis* are not palatable, necessitating a purification or extraction step, which may add to the cost of production. An advantage of producing pharmaceuticals in potato is that the tuber is a good tissue for production and storage of proteins. Nevertheless, potato may require cooking to improve its palatability. Arakawa et al. (1998) have shown that boiling CT-B generating transgenic potato denatures up to 50% of the antigen. By contrast, although yields of cereal grains such as wheat, rice and maize are less abundant than that of the green tissues of

tobacco and alfalfa, high seed production in these plants makes scaling up quite easy. Unlike proteins synthesized in vegetative plant tissues, seed storage proteins are compartmentalized in protein bodies, specialized vacuoles in mature seed. These provide a stable environment devoid of significant amounts of enzyme activity prior to germination. Another advantage of producing functional proteins in cereal grains is the stability of protein during long-term storage. Levels of scFV antibody in rice seeds or tubers did not show a significant decline after storage at room temperature for 6 months (reviewed by Daniell et al., 2001).

Maize is a convenient and practical crop for production of an edible vaccine. It has high yield, is a major ingredient in livestock feed and is a staple food in many countries. Additionally, maize does not require extensive heat during processing, which might lead to denaturation of antigens, among other advantages.

Work by Mason et al. (1998) and Tacket et al. (1998) showed that potato produced LT-B subunits were capable of inducing both systemic and mucosal antibodies in mice and humans respectively. In this study we investigated the ability of maize-synthesized LT-B to retain properties of the native bacterial protein such as antigenicity, pentameric structure, G_{M1} ganglioside receptor binding capacity, and immunogenicity in mice. We used maize the gamma zein promoter (Marks et al., 1985), a seed endosperm specific promoter, to direct LT-B expression in maize kernels. We demonstrated that maize synthesized LT-B folded properly into pentameric structures with affinity for G_{M1} gangliosides, like the native LT-B. When fed to mice, the maize synthesized LT-B stimulated a protective immune response against diarrhea inducing toxins LT and its closely related cholera toxin (CT). In addition, feeding transgenic maize to mice did not result in the induction of tolerance to LT-B, as shown by the presence of significant systemic and mucosal anti-LT-B and CT-B antibodies.

Our results indicated that maize could be efficiently transformed to produce, accumulate and store fully assembled and functional antigen.

Material and Methods

Cloning and bacterial transformation

The enzymes *Xho* I and *Eco*R I were used to cut out the 1.15 kb fragment including the tobacco etch virus (TEV) 5' untranslated region that mediates enhancement of translation initiation (Gallie et al., 1995), the synthetic LT-B gene (Mason et al., 1998) and the soybean vegetative storage protein (VSP) terminator (Mason et al., 1993) from the plasmid pTH210 (H. Mason, unpublished). This fragment was cloned in front of the maize 27 kD gamma zein promoter (Marks et al., 1985) in a pUC19 vector to give the plasmid pRC4 (Figure 1). The resultant plasmid pRC4 was sequenced to ensure correct orientation and fidelity of ligation junctions. DNA for maize transformation was obtained using the Qiagen (Qiagen GmbH, Germany) Maxiprep kit according to the manufacturers instructions.

Maize transformation

Embryogenic maize Hi II callus was transformed using microprojectile bombardment as described by Frame et al. (2000). Briefly, the plasmid pRC4 (Figure 1) encoding the synthetic LT-B gene was co-bombarded with a selectable marker gene construct, pBAR184. This construct contains an ubiquitin promoter/*bar* gene cassette that confers resistance to the herbicide bialaphos (Frame et al., 2000). Herbicide resistant calli were analyzed using the polymerase chain reaction (PCR) for presence of the LT-B gene cassette. Calli of transgenic events were regenerated and plants brought to maturity in the greenhouse

DNA extraction from maize callus and leaf tissue

Plant DNA was extracted from callus as follows: 0.1-0.25 g of callus were collected in a 1.5 ml eppendorf tube and ground up with a plastic Kimble pestle (Fisher Scientific, Pittsburgh, PA, USA) in 400 µl extraction buffer [200 mM Tris-Cl (pH 7.5), 250 mM NaCl, 25 mM EDTA and 0.5% SDS]. An equal volume of phenol chloroform isoamyl alcohol (25:24:1, v/v/v) was added and mixed well by inverting the tube several times. The tubes were centrifuged at maximum speed on a bench top centrifuge for 10 minutes. The aqueous phase was transferred to a clean eppendorf tube and the DNA precipitated with 2-propanol and washed with 70% ethanol. Leaf genomic DNA was extracted from R_1 maize plants using a cetyltrimethylammonium bromide (CTAB) protocol, as described by Murray and Thompson (1980). Three grams of fresh leaf tissue were ground to a fine powder in liquid nitrogen and resuspended in 25ml of CTAB buffer (1% CTAB, 50 mM Tris, 0.7 M NaCl, 1 mM phenathroline, pH 8.0) supplemented with 0.5% beta-mercaptoethanol just before use. Samples were incubated for 30 minutes at 65°C, then allowed to cool to room temperature. Twenty milliliters of chloroform : isoamyl alcohol (24:1, v/v) were added and the samples mixed by inverting on an orbital shaker for 10 minutes. The tubes were centrifuged at 2,500 g and the aqueous phase was collected. DNA was precipitated from the aqueous phase in 2/3 volume of 2-propanol, with gentle inversion, and removed with a glass hook. The DNA was washed for 30 minutes at room temperature in 80% ethanol plus 15 mM ammonium acetate. The liquid was removed and DNA air-dried and dissolved in TE (10 mM Tris, 1 mM EDTA, pH 8.0).

Polymerase Chain Reaction

Transgenic calli were initially selected on the basis of resistance to bialaphos and the presence of the LT-B gene was confirmed by PCR analysis. The forward and reverse primers, respectively, used were, LT-B-PCRF1 (5'-atc gat aca aaa caa acg aat ctc aag c-3') and LT-B-PCRR1 (5'-cca tgg cgt gga ttt tat gac att tta t-3'). These primers amplify an 842 bp fragment from the TEV leader to part of the VSP terminator, including the LT-B gene. PCR reactions were carried out in a total volume of 50 µl containing 50-250 ng of maize callus genomic DNA, 5 µl of 10X PCR buffer, 2 µl of dNTP mix (containing 10 mM of each dNTP), 12 µl of each primer (2.5 µM stock), and 1 µl of Biolase Taq Polymerase (Biolase USA Inc., City, NJ, USA). Reaction conditions were as follows: Initial PCR activation (95°C, 3 minutes) was followed by 30 amplification cycles (denature, 94°C, 30 seconds; annealing, 60°C, 30 seconds, and extension, 72°C, 45 seconds) and a final extension step at 72°C for 5 minutes.

Southern blot analysis

Ten micrograms of leaf genomic DNA were digested with restriction enzyme *Nco*I at 37°C overnight and separated on a 0.8% agarose gel. Linearized DNA gel blot analyses (Sambrook et al., 1989) were conducted on DNA samples using the ^{32}P-labeled LT-B gene fragment as probe (Figure 1).

Protein extraction from maize kernel

Individual mature dried kernels frozen in liquid nitrogen were ground to a fine powder in a mortar and pestle. The fine powder was transferred into a 1.5 ml eppendorf tube

and weighed. For ELISA analysis, 25 mM sodium phosphate buffer (pH 6.6) containing 100 mM NaCl, 10 mM EDTA and 0.5% Triton X-100 (v/v) was added at the rate of 10 µl buffer per milligram of maize powder. For Western analysis, extraction buffer [200 mM Tris-HCl, (pH 8.0), 100 mM NaCl, 400 mM sucrose, 10 mM EDTA, 14 mM 2-Mercaptoethanol, 0.05% Tween-20] was added at the rate of 10 µl buffer per milligram of maize powder. The powder was soaked for one hour on a vortex shaker at room temperature and centrifuged at maximum speed on a bench top centrifuge for 15 minutes to remove insoluble debris. Protein concentrations were determined as total soluble protein (TSP) by the Bradford Assay (Bradford, 1976) with the BioRad (Hercules, CA, USA) protein dye concentrate using a standard curve derived from bovine serum albumin (BSA).

To identify transgenic R_0 events expressing LT-B, individual kernels from each ear (two ears per event) were analyzed by a partial destruction method, where kernels were partially drilled to remove part of the endosperm without damaging the embryo as described by Sangtong et al. (2001). Protein was extracted from endosperm as described for whole kernels. Kernels expressing LT-B were planted to give the R_1 generation.

Quantitation of LT-B expression in maize

LT-B expression in maize was determined using ganglioside dependent enzyme linked immunosorbent assay (ELISA). Reagents and antisera for the ELISA were obtained from Biogenesis Inc. (Kingston, NH, USA) and KPL (Gaithersburg, MD, USA). Volumes of 50 µl were used throughout all ELISA assays unless otherwise specified. Wells were washed three times between each step using 300 µl of phosphate buffered saline Tween-20 [PBST; 0.01 M Na_2HPO_4, 0.003 M KH_2PO_4, 0.1 M NaCl, (pH 7.2), 0.05% Tween-20 (v/v)]. ELISA

was carried out at 37°C throughout the process unless indicated otherwise.

Microtiter plates (Costar 3590, Fisher Scientific, PA, USA) were coated with Type III G_{M1} gangliosides (1.5 μg/well) from bovine brain (G2375, Sigma, St Louis, MO) in sodium carbonate coating buffer [15 mM Na_2CO_3, 35 mM $NaHCO_3$, 3 mM NaN_3 (pH9.6)] for one hour. The plates were blocked to prevent non-specific antibody binding with 5% dry milk (w/v, DM, DIFCO, Becton Dickinson, MD, USA) for one hour at room temperature. The protein extracts were added to the ganglioside coated microtiter dish, incubated for one hour, followed by incubation with goat anti-LT-B antiserum (1:1500 dilution, Biogenesis) for one hour. Rabbit anti-goat alkaline phosphatase conjugate [1:2500 dilution in 1% DM (w/v) in PBS] was added, and the dish incubated one hour. This was followed by addition of phosphatase substrate, 1 mg ml^{-1} ρ-nitrophenyl phosphate (Sigma) in alkaline phosphatase buffer (pH 9.5). Absorbance at 405 nm was measured spectrophotometrically over a two-hour period in a Dynatech MRX Plate Reader (Dynex Technologies, VA, USA). Sample wells were blanked against non-transgenic maize protein extracts and all measurements were performed in duplicate. Raw ELISA data were converted to ng ml^{-1} of LT-B of total soluble protein by reference to an ELISA standard curve constructed using purified bacterial LT-B (kindly provided by John Clements, Tulane University, LA, USA).

Immunoblot analysis

A 10-20 μl aliquot containing 50-100 μg of total protein from maize kernels was analyzed by SDS-PAGE (Laemmli, 1970). Protein samples were either boiled for five minutes at 95°C or loaded directly on the gel without heat treatment. The separated proteins were transferred to a 0.45 μm nitrocellulose membrane using the BioRad Transblot apparatus

according to the manufacturer's instructions. Proteins were evaluated for presence of monomeric or oligomeric LT-B in boiled and non-boiled samples, respectively, by Western Blot analysis. The analysis used goat anti-LT-B antiserum and an alkaline phosphatase conjugated anti-goat IgG (KPL) at 1:3000 and 1:10,000 dilutions, respectively, using the procedures of Blake et al. (1984).

Preparation of Feeding Pellets

Kernels harvested from individual ears of transgenic R_1 plants were milled separately in a coffee grinder (Braun, Type 4041: Model KSM2). The LT-B content of each batch of maize powder was determined by ELISA and results are shown in Table 1. Maize powder from self-pollinated transgenic event P77-2 was selected for feeding mice. The LT-B concentrations in milled maize powder ranged between 10 to 13 $\mu g\ g^{-1}$. Non-transgenic maize kernels were added to adjust the concentration of LT-B to 10 $\mu g\ g^{-1}$. To prepare the pellets for feeding mice, a fixed amount of maize meal was weighed out and sterile distilled water added to make firm dough. The dough was then molded into a cylinder (Figure 5) using a 3 ml disposable syringe barrel with the Leur tip cut off. The cylindrical dough was cut into blocks (2 cm in length) that weighed 0.9-1.2 gram each when dried. The pellets were allowed to air-dry overnight and then weighed again prior to feeding to ensure that they weighed as close to one gram as possible. One extra pellet was prepared for each feeding, to be assayed for LT-B content at the end of the experiment. Non-transgenic maize meal was treated in a similar manner to make negative control pellets. To make the positive control, the non-transgenic maize powder was weighed out and mixed with bacterial LT-B in sterile water. Each dried pellet weighed one gram and contained 10μg of the bacterial LT-B

protein.

Mice feeding

Five-week old female inbred BALB/c mice were obtained from Harlan (Indianapolis, IN, USA) and were allowed a two-week adjustment period with reverse light dark cycle prior to onset of experiment. The mice were kept in the Iowa State University animal facility. All animal procedures were approved by the Iowa State University Laboratory Animal Resources Committee on Animal Care prior to experimentation.

Mice were fasted 12 hours prior to feeding with water *ad libitum*. Three feed categories were used for this experiment, non-transgenic B73 maize pellets, non-transgenic maize spiked with 10 $\mu g\ g^{-1}$ bacterial LT-B and LT-B expressing transgenic maize pellets. These feed types are referred to in the figures as Negative, Spiked and Transgenic respectively. Twelve mice were used per feed category. Two mice were placed in each cage and two pieces of the appropriate maize pellets were placed in a 15 x 60 mm dish on either end of the cage just before lights out. Bedding in the feeding cages was removed to ensure that all pellets were completely consumed during feeding. The dishes were then removed three hours later and the mice returned to their main cages with their normal rodent food. At the end of the three-hour period the mice had consumed all the maize pellets supplied. Mice were fed on days 0, 3, 7, and 21.

Serum and fecal sample preparation

For anti-LT-B and anti-CT-B antibody quantity determination, fecal pellets were collected and blood (approximately 100 µl) obtained once a week prior to fasting and feeding

and once before euthanasia (days -1, 6, 13, 20 and 27). Mice were not bled prior to feeding at day 3. Mice were bled sephanously. Immediately after collection, the blood sample from each animal was centrifuged at 17 000xg in a microcentrifuge for 10 minutes to separate serum from clotted blood, and the sera collected and stored at -20°C until assayed. For ELISA evaluation of anti-LT or CT antibodies, sera collected on day 27 were diluted in PBS at 1:50 ratio for IgG assays and 1:20 ratio for IgA assays. Lower dilutions (1:10 to 1:25 for IgG and 1;10 to 1:20 for IgA) were used for sera from earlier bleeds with lower antibody titers. Fecal pellets were stored at -80°C until assayed. Fecal pellets were removed from -80°C and lyophilized for 36-48 hours in open eppendorf tubes set upright in a Virtix Freezemobile 12LX lyophilizer. Dry fecal pellets were weighed, and PBS buffer supplemented with 0.2 mg ml^{-1} (w/v) trypsin inhibitor, 12.5% sodium azide (w/v) and 1 mM PMSF was added at 10µl for every milligram of dry fecal matter. Samples were soaked in buffer overnight at 4°C, and then centrifuged at 17,000xg on the micro centrifuge for 10 minutes and the supernatant removed for ELISA analysis as described below.

Determination of anti-LT-B and anti-CT-B antibodies

Reagents and antisera for the ELISA were obtained from Sigma. Antisera for ELISA were diluted in PBS buffer as described above. For anti-LT-B or anti-CT-B determinations, microtiter plates were pre-coated with 1.5 µg of mixed gangliosides (Type III form Bovine, Sigma G2375) per well diluted in sodium carbonate coating buffer (see above) for 1 hour. This was followed by incubating with 1µg/well purified LT-B (John Clements) or CT-B (Sigma C9903) at 37°C for 1 hour. Plates were blocked with 5% DM (w/v) in PBS for one

hour at room temperature. Diluted serum samples were added and the dishes incubated at 37°C for one hour. Anti-LT-B or anti-CT-B IgG levels in serum were determined by incubating with rabbit anti-serum against mouse IgG conjugated to alkaline phosphatase (Sigma A2418, diluted 1:7000 in 1% dry milk) at 37°C for 1 hour. IgA antibodies were similarly determined in parallel using rabbit anti-serum against mouse IgA conjugated to alkaline phosphatase (Sigma A4937, also diluted 1:7000 in 1% dry milk). Plates were read over a two-hour period as described above. Extracted fecal samples were measured without further dilution. Values for IgG and IgA were determined from a standard curve with purified mouse myeloma proteins [MOPC 21γG1, MOPC 315 γA(IgAλ2)] obtained from Sigma.

Patent mouse assay

The patent mouse assay (Guidry et al., 1997) was used to determine protection from toxin challenge. It is a modification of the sealed adult mouse assay (Richardson et al., 1984). The mice were challenged with LT (John Clements) and CT (Sigma C3012) toxins. The mice were fasted for 12 hours prior to the challenge with water available *ad libitum* and orally gavaged with 200 µl of PBS (pH 7.2) containing (25 or 50 µg) LT or CT toxins. After toxin administration (three hours), the mice were euthanized by carbon dioxide inhalation, dissected and the gut removed from the duodenum to the anus. The gut, with the fat pad and the mesentery left intact, and the remaining body were weighed separately. The gut/carcass ratio (Richardson et al., 1984) was then calculated to determine the extent of toxin induced water influx into the gut.

Statistical Analysis

Data was analyzed using the package Statistix for Windows, Version 4.0 (Analytical Software, Tallahassee, FL) and the Students t-test. The results are reported as mean values ± standard deviation (SD).

Results

Analysis of transgenic maize lines

A plant optimized coding sequence of LT-B (sLT-B) from *E. coli* strain H10407 (Yamamoto et al., 1982) was designed and synthesized by Mason et al. (1998). Construct pRC4 (Figure 1) was introduced into a maize hybrid line Hi II via particle bombardment as described (Frame et al., 2000). Transformed maize callus lines were analyzed by polymerase chain reaction (PCR) to confirm the presence of the sLT-B gene. Because an endosperm specific promoter was used, it was expected that the transgene would only express in endosperm of transgenic maize. Therefore, herbicide resistance and presence of the LT-B gene in PCR analysis were the criteria for selecting transgenic calli. Sixty independent herbicide resistant events were analyzed, forty-seven were found to contain the LT-B gene. Three plants per event were regenerated from 20 of these events and grown in the greenhouse to maturity.

Nineteen of the twenty regenerated transgenic events were fertile. These plants were crossed with pollen from the non-transgenic inbred line B73. Mature transgenic kernels were analyzed for expression of the LT-B gene using ganglioside dependent ELISA as described by Haq et al. (1995) and in Material and Methods. The LT-B expression in R_1 seed from these events is summarized in Figure 2. LT-B expression ranged from less than 0.01% to

greater than 0.05% LT-B of total soluble protein as shown. Nine events expressed less than 0.01% LT-B of total soluble protein, eight events had LT-B levels between 0.02-0.05% and two events shown expression above 0.05%.

Six events were chosen to increase seed for further analysis on the basis of seed availability and level of expression of LT-B. Seeds were planted from the expressing R_0 events to give R_1 generation plants, which were grown to maturity in the greenhouse. These plants were either self- pollinated or pollinated with non-transgenic inbred line B73 or hybrid line Hi II (Table 1).

Southern analysis was carried out on selected events P77–2, –7 and –9 using leaf material of R_1 plants to determine transgene copy number (Figure 3). Since *Nco*I cut only once in the transgene cassette, the hybridization banding pattern should give an estimation of the number of LT-B gene copies present in the maize genome. From the number and intensity of bands in the autoradiograph, it can be concluded that all three events had multiple gene insertions and possible tandem repeats in the same insertion site.

Immunoblot analysis was performed on five transgenic events using the R_2 kernels to detect the presence of the assembled LT-B subunits. The molecular weight of an LT-B monomer is 11.6 kD. However, only properly assembled LT-B subunits in a pentameric structure (55 kD) have GM_1 binding capacity and hence native antigenic function (de Haan et al., 1998). As reflected in Figure 4, maize synthesized LT-B from R1 seeds of events P77–2, –7, –17 and –18 had similar molecular weight to the bacteria derived LT-B in either pentameric form (Figure 4A) or monomeric form (Figure 4B). No LT-B protein was detected from event P77-1 by western analysis. ELISA analysis also indicated that the LT-B expression of this event was low (Table 1).

For mice feeding experiments, dry kernels from each R_2 ear were harvested and ground to a fine meal. LT-B of the meal was assayed using ELISA (Table 1). Since the LT-B transgene in the R_2 seed population was segregating at a ratio of either 3:1 (for selfed plants) or 1:1 (for out-crossed plants, data not shown), the maize meal obtained from these events were a mixture of LT-B expressing and non-expressing seeds.

The expression observed in the meal of six events ranged from 0.004 - 0.19% LT-B of total soluble protein (Table 1). In event P77-9, the LT-B expression increased 10-fold compared to the level detected in their R_1 kernels. The LT-B expression level varied not only between independent events, but also between ears within one event. Event P77-2 was used for the mice feeding experiment for its adequate LT-B expression and sufficient seed yield at the time of experiments. A total of five P77-2 ears from selfed plants were pooled for the feeding experiment.

Anti-LT-B antibody response in mice

Mice were fed with maize pellets (Figure 5) containing 10 μg of maize synthesized or bacterial LT-B on days 0, 3, 7 and 21. Figure 6 depicts the anti-LT-B antibody responses detected in the blood and fecal pellets of orally immunized mice. Results are presented as mean±SD. Serum anti-LT-B IgG levels in blood were first detected on day 13 following initial immunization. For mice fed with both transgenic maize pellets and maize pellets spiked with the bacterial LT-B, this was reflected as a late stage primary response in (Figure 6A) as antibody class switching to IgG occurred. A secondary immune response is observed as a peak mean antibody response (5.7±1.1 mg ml^{-1}) at day 27 (one week after the fourth feeding). The IgG concentration is about double the amount observed in the primary

response. The response of the transgenic maize fed group was similar to that observed in mice immunized with bacterial LT-B, which also peaked (5.8±0.7 mg ml^{-1}) at day 27. There were no changes in specific antibody production in mice fed with maize pellet made from non-transgenic maize (negative group) through out the experiment.

Fecal secretory IgA plays an important role in attenuation of toxin effects, possibly more so than the serum IgG and IgA because it is secreted in the gut at the point of contact with the toxin. Figure 6B shows the anti-LT-B IgA concentrations detected from fecal pellets of immunized mice. The secretory IgA of transgenic maize-fed mice increased gradually over the course of the experiment and peaked (278±50 μg g^{-1} of dry fecal matter) at day 27. Mice fed with maize spiked with the bacterial LT-B also developed anti-LT-B IgA in their fecal pellets. However, the secondary response of these mice was significantly lower (149±21 μg g^{-1} dry fecal matter, $p<0.001$) than that of transgenic maize-fed mice. No substantial production of fecal anti-LT-B IgA was detected in mice fed with non-transgenic maize pellet.

The serum anti-LT-B IgA was determined during the course of the experiment and the results are shown in Figure 6C. Both the maize synthesized and bacterial LT-B protein induced serum anti-LT-B IgA in mice, and both show clearly defined primary and secondary antibody responses. However, transgenic maize induced a significantly higher serum IgA (428±68 μg ml^{-1}, $p<0.001$) on day 27, compared to the bacterial LT-B (250±36 μg ml^{-1}), which also induced a significantly higher serum IgA response than the negative control ($p<0.001$).

Anti-CT-B antibody response in immunized mice

The cholera toxin (CT) is physically, chemically and antigenically similar to LT (Dickinson and Clements, 1996). To investigate whether mice immunized with maize synthesized or bacterial derived LT-B protein would also generate cross-reacting antibodies that would bind CT-B, we performed anti-CT-B antibody analysis on mouse serum and fecal samples (Figure 7). Figure 7A shows the concentration of serum anti-CT-B IgG over the course of the experiment. In contrast to anti-LT-B IgG, no marked responses to primary immunization were detected in mice fed with either transgenic maize or bacterial LT-B spiked maize pellets. Elevated serum anti-CT-B IgG antibodies were detected at day 27. The IgG level in transgenic maize-fed mice was significantly higher (2.3±0.8 mg ml^{-1}) than that of LT-B spiked maize-fed mice (1.0±0.5 mg ml^{-1}). No anti-CT-B IgG antibodies were detected in the negative control mice fed with non-transgenic maize pellets.

Figure 7B shows the cross-reacting anti-CT-B fecal IgA antibody production. Although some anti-CT-B IgA antibody was detected in mice fed with either maize synthesized or bacterial LT-B over the course of experiment, they were not very high above the background. Mice fed with the control maize pellets (Negative group) also display measurable production of anti-CT-B antibody. The fecal anti-CT-B cross-reacting antibodies are only marginally higher ($p<0.02$) in mice immunized with transgenic maize at the end of the experiment (day 27).

Figure 7C shows the cross-reacting serum anti-CT-B IgA antibodies at the end of the experiment (day 27). The antibody production over the course of immunizations could not be tracked over the course of the experiment because of limitations in serum availability from earlier bleeds. Transgenic maize pellet induced a higher response (196±69 $\mu g\ ml^{-1}$, $p<0.01$)

compared to the bacterial LT-B spiked maize pellet in mice. Interestingly, spiked LT-B maize pellets did not induce significantly higher (69±40 μg ml^{-1}) serum anti-CT-B IgA in mice compared to non-transgenic negative control pellets.

Response of immunized mice to challenge with toxins LT and CT

To investigate whether maize synthesized LT-B provides protection against toxin challenge, we performed the patent mouse assay (Guidry et al., 1997) in the orally immunized mice (Figures 6 and 7), using LT or CT to challenge the mice. These toxins activate adenylate cyclase and result in fluid secretion into the lumen of the bowel. The fluid secretion exceeds the bowel's capacity to reabsorb it and therefore it accumulates (Mason et al., 1998). The movement of fluid from the body into the lumen of the bowel decreases the weight of the carcass and increases the relative weight of the gut. The gut/carcass ratio therefore changes in a dose dependent fashion. In a toxin dose-response experiment (Figure 8A), we measured the gut/carcass ratio of non-immunized mice gavaged with two levels of LT and CT (25 and 50 μg). Significant fluid influx was detected in mice gavaged with both levels of LT and CT toxins when compared to mice gavaged with phosphate buffered saline (PBS) as a negative control ($p<0.02$ for 25 μg and $p<0.001$ for 50 μg). A significant difference in gut/carcass ratio between LT and CT ($p<0.02$) was observed when 50μg of toxin were administered. The CT was significantly more potent than the LT as expected (Bowman and Clements, 2001). There was no significant difference in fluid influx observed between LT and CT at 25 μg/dose level.

Figure 8B shows the results of the patent mouse assay performed on day 28 of the experiment. Compared to mice that were fed with non-transgenic maize, mice immunized

with LT-B expressing transgenic maize had reduced gut/carcass ratio when challenged with 25 μg LT toxin. The gut/carcass ratios for transgenic maize-fed mice challenged by LT toxin was not significantly different from non-transgenic maize-fed mice gavaged with PBS only (0.109±0.001 vs 0.095±0.0002), indicating that mice orally immunized with transgenic maize were protected from challenge with 25 μg LT toxin. Mice fed with LT-B-spiked maize also showed a similar reduction in the gut/carcass ratio. There was significant fluid influx in mice that were given the non-transgenic maize meal compared to the PBS control group (0.130 vs 0.095, $p<0.01$). The gut of mice from all feed categories gavaged with PBS (shown as PBS group in Figure 8B) did not accumulate fluid indicating that the gavage procedure itself did not cause an inflammatory response resulting in fluid accumulation.

Similar results were observed when orally immunized mice were challenged with 25 μg CT-B (Figure 8B). Mice fed with either transgenic maize meal and LT-B-spiked maize meal also had reduced fluid influx compared to mice fed with non-transgenic maize and gavaged with CT toxin. The gut to carcass ratio for mice in these two categories (transgenic maize and spiked) was not significantly different from mice fed with negative maize and gavaged with PBS (0.095±0.0002).

Discussion

We have demonstrated that a synthetic gene encoding *E. coli* LT-B can be successfully expressed in transgenic maize seed and accumulating in pentameric G_{M1} binding form. Oral immunization of BALB/c mice with maize synthesized LT-B induced elevated IgA and IgG serum antibodies and elevated fecal IgA against diarrhea-inducing toxins LT and CT. In addition, transgenic maize-fed mice challenged with LT or CT toxins displayed

reduced gut/carcass ratios that were not significantly different from the PBS challenged negative control group, indicating that the maize synthesized LT-B has biological and immunological functions comparable to the native LT-B protein that can protect immunized mice from LT or CT toxins challenge.

In this study, we inserted into the maize genome a synthetic LT-B gene that was optimized for expression in both potato and maize (Mason et al., 1998). To achieve a high level of gene expression, we used the 27 kD maize gamma zein promoter to drive the transcription of the sLT-B gene. The 27 kD gamma zein promoter is one of the strongest seed specific promoters in maize, and zeins comprise 50-60% of the total seed storage proteins (Marks et al., 1985). In contrast to other plant tissues, seeds provide a relatively dry and stable environment for protein storage. Seeds also present potentially practical and protective delivery vehicles for targeting edible vaccines into the gut because of their palatability and high protein content. The antigenic protein may be delivered within protein bodies, and therefore not in readily soluble form. Presentation of proteins in soluble form has been shown to be conducive to oral tolerance (Simmons et al., 2001). The initial level of LT-B expression in R_1 seed ranged from less than 0.01% to 0.07% LT-B of total soluble proteins (TSP). Considerable variation in LT-B expression was observed between and within each independent event. Southern analysis was conducted on the best performing events P77-2, -7, and 9. The events have multiple transgene copy numbers and variable insertion patterns. Gene copy numbers and the relative positions of transgene insertion in the plant genome (position effects) may be factors that affect level of transgene expression (Christou et al., 1996).

Compared to the LT-B expression in R_1 seed, we observed an increased LT-B

expression in the R_2 seed for the same event. LT-B expression ranged from 0.004 – 0.2 % of TSP. The reason for this increase is not clear. However, this phenomenon has also been reported by Hood et al. (1997), they observed a similar increase in avidin gene expression was observed in transgenic maize in subsequent generations from R_1 generation. We observed but did not follow up events with decreased LT-B expression over the generation. It is worth noting that the LT-B levels obtained from R_2 seed was from a mixture of segregating seed population from each event, with expression ratio of 3:1 for selfed and 1:1 for out-crossed plants. It is conceivable that the LT-B yield would be higher if only expressing kernels had been included in the meal.

The ganglioside dependent ELISA used to determine level of LT-B expression demonstrated the ability of maize synthesized LT-B to bind G_{M1} gangliosides, a property of native pentameric LT-B (Dickinson and Clements, 1996). Our ELISA results indicated that the maize produced LT-B was similar to the bacterial LT-B that properly folded and assembled into pentamers. Immunoblot analysis indicated that the maize synthesized LT-B was similar in molecular weight to the bacterial LT-B, the non-boiled form was oligomeric and dissociated into monomers upon boiling.

Mice fed with LT-B expressing transgenic maize developed serum and mucosal antibodies that are specific to LT-B and cross-react with the closely related CT-B. It is difficult to accurately quantify the secretory IgA in the gut as the amounts measured in fecal pellets may be a reflection of the remaining antibody after degradation by microbes that live in the gut, among other processes. Serum IgA, while not involved in protection of host from invading pathogens and toxins, was used to corroborate the differences in effect of the different feed types (compare Figures 6B and C).

Interestingly, compared to spiked LT-B-fed mice, we observed a significantly higher level of anti-LT-B IgA in fecal and serum samples of transgenic maize-fed mice (Figure 6B and C) especially at day 27 when the maximum amount of antibody was expected. One possible explanation could be that the maize synthesized LT-B is more stable within the maize tissues and was released slowly as the meal was digested. The spiked bacterial LT-B, on the other hand, could be more vulnerable to proteolytic degradation during the process of pellet preparation and feeding as it is merely mixed in with regular maize meal. An alternative explanation could be that there was more LT-B in transgenic maize than the ELISA assay indicated depending on the fineness of the meal. We observed that the finer the maize meal was ground, the higher the LT-B yield we measured, suggesting that a finer maize powder allows better protein extraction. However, very fine maize meal did not mould well to make pellets for feed, so we used an average textured meal to make the pellets for feeding.

Cross-reacting anti-CT-B antibodies could also be detected in mice fed with LT-B expressing transgenic maize or LT-B-spiked maize. There were overall more LT-B binding antibodies compared to CT-B binding IgG and IgA (compare Figure 6A with 7A and Figure 6B with 7B). This is not unexpected since the B subunits from the two toxins share some common epitopes but are not identical. There is a 20% difference in nucleotide and amino acid sequence levels (Dallas and Falkow, 1980). Therefore among antibodies produced in response to LT-B immunization, only those produced to the common epitopes will cross-react with CT-B.

It is important that an oral vaccine should elicit an immune response in the gut. It has been shown that in many cases, it is possible to prevent the initial infection by stimulating

production of mucosal secretory IgA against specific virulence determinants on the pathogen. Specific secretory IgA prevents interaction of the pathogen with the mucosal surface by blocking attachment and/or colonization to prevent invasion of host cells or by neutralizing surface acting toxins (Dickinson and Clements, 1995). We show here that the maize synthesized LT-B stimulates secretory IgA in the gut and is therefore functional in that regard.

We have demonstrated that mice orally immunized with transgenic maize or LT-B spiked maize have reduced fluid influx in their guts (gut/carcass ratio) compared to the negative maize-fed mice, indicating that the LT-B-immunized mice were protected from the toxin challenge. In this study, 25 μg of LT used for challenge was determined previously (Mason et al., 1998). It was found that in a dose response curve, the gut/carcass ratio reached a plateau at 0.16 at a dose of 25 μg of LT (Mason et al., 1998). This level of LT and CT used for the challenge was quite substantial for mice considering their low body mass. For comparison, in one human trial, 25 μg of native CT administered orally was shown to elicit a 20-liter cholera purge in human volunteers (Levine et al., 1993), while 25μg of LT administered with a whole cell B subunit cholera vaccine was shown to elicit up to 6 liters of fluid (Bowman and Clements, 2001).

The gut/carcass ratio used for the efficacy assessment is toxin dose dependent. In our patent mouse assay, challenge with 25 μg of toxin showed attenuation of both LT and CT toxin effects in orally immunized mice (Figure 8B). When 50 μg of toxin were used, the gut/carcass ratio of the transgenic maize fed group was no longer different from that of toxin challenged negative maize group (data not shown), suggesting that at this level of toxin,

orally immunized mice were not protected from these diarrhea-inducing toxins.

While we detected more LT-B binding IgG and IgA compared to CT-B binding antibodies, there was no significant difference in protection between LT and CT challenged mice. The apparent low anti-CT-B antibody concentrations observed could merely be a reflection of the limit of detection of the ELISA. Data for anti-CT-B binding antibodies showed more variability compared to anti-LT-B serum data, especially at lower titers. This may be an artifact in measuring these antibodies. Alternatively, this could be due to the simple reason that there are not as many cross-reacting anti-CT-B antibodies, and the few that are present are adequate to attenuate the effect of CT.

Despite the attractiveness of mucosal vaccination, mucosally administered proteins are frequently not immunogenic (Bowman and Clements, 2001) and require more antigen relative to parenteral vaccination (Simmons et al., 2001). A number of strategies have been developed to facilitate and enhance the immune response obtained after mucosal vaccination and to prevent induction of oral tolerance. The use of adjuvants is one such strategy and LT-B has been shown to be a potent one. Millar and co-workers (2001) showed that contrary to previous opinion (reviewed by Dickinson and Clements, 1996), the non-toxic subunits LT-B and CT-B are potent oral immunogens. These subunits avoid tolerance induction when administered mucosally and generate strong serum and mucosal responses (reviewed by Snider, 1995). They are arranged in a pentameric ring and contain five receptor-binding pockets for high avidity association with cellular G_{M1} gangliosides. We have demonstrated here that maize synthesized LT-B has a mitigating effect against both LT and CT toxins. Protein less immunogenic than LT-B may not stimulate an immune response when orally administered in an edible transgenic plant or may even induce tolerance against itself. LT-B

can therefore be co-expressed in the same plants with the less immunogenic vaccines to achieve a more vigorous immune response. As an adjuvant, LT-B could therefore play a key role in providing safe and cost effective mucosal immunization against enteric pathogens, which can be effectively controlled by recombinant subunit vaccines.

LT-B could be also used as a cost effective carrier for induction of an increased mucosal response to antigens to which it is chemically or genetically conjugated. Because of its ganglioside binding properties, LT-B has the capacity to bring conjugated antigens in contact with the mucosal system, making oral vaccination more efficient. Increased immunogen concentration in the mucosal lymphoid tissues may reduce the requirement for high levels of antigen biosynthesis in transgenic plants.

Maize synthesized LT-B did not induce tolerance as indicated by the production of antibodies over the vaccination period. We have shown that maize synthesized LT-B conferred protection against both LT and its closely related CT. Maize is highly palatable and as such, a reasonable crop for vaccine production. The fasted mice easily consumed one gram of pellet containing LT-B protein within three hours. Production of LT-B in kernels and palatability of maize pellets made it easier and convenient to administer an oral vaccine in maize relative to other plants. Feeding periods for administration of adequate doses with potato reported by Mason et al. (1998), were quite prolonged, up to 24 hours. Because of the high level of expression of the LT-B that could be achieved in maize, only one gram was required to administer an adequate dose (10 μg) of LT-B.

Recent research has demonstrated the potential of plant-derived antigens for immunization against infectious agents that invade through mucosal surfaces (Tacket et al., 1998). Maize is a major feed ingredient and could potentially play a key role in the

production of edible vaccines for livestock. Oral vaccine delivery from maize seed would be convenient, practical and would allow long-term storability and stability of the proteins. Storage of antigen in protein bodies is expected to protect it from immediate enzymatic degradation and from the acidic environment in the stomach (Simmons et al., 2001). In addition, it will allow the protein to be released slowly. This is advantageous because delivering oral vaccines in soluble form is conducive to oral tolerance. Questions under further investigation in our laboratory include increased expression to address dosage requirement and dosage stability in grain.

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References

Arakawa, T, Chong, DKX, Merrit, JL, Langridge, WHR (1998) Expression of Cholera toxin B subunit oligomers in transgenic potato plants. *Transgenic Research* **6**: 403-413.

Blake MS, Johnston KH, Russell-Jones GJ, Gotschlich EC (1984) A rapid sensitive method for detection of alkaline phosphatase conjugated antibody on Western blots. *Annals of Biochemistry* **136**: 175-179.

Bowman, CC, Clements, JD (2001) Differential biological and adjuvant activities of Cholera toxin and *Escherichia coli* heat –labile enterotoxin hybrids. *Infection and Immunity* **69**: 1528-1535.

Bradford, MM (1976) A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry* **72**: 248-254.

Christou, P (1996) Transformation technology. *Trends in Plant Sciences* **1**: 423-431.

Dallas, WS, Falkow, S (1980) Amino acid sequence homology between homology cholera toxin and the *Escherichia coli* heat labile toxin. *Nature* **288**: 499-501.

Daniell, H, Streatfield, SJ, Wycoff, K (2001) Medical molecular farming: production of antibodies, biopharmaceuticals and edible vaccines in plants. *Trends in Plant Science* **6**: 219-226.

de Haan L, Feil IK, Verwej WR, Holtrop, M, Hol, WG, Agsteribbe, E, Wilschut, J (1998) Role of GM1 binding in mucosal immunogenicity and adjuvant activity of the *Escherichia coli* heat labile enterotoxin and its B subunit. *Immunology* **94**: 424-430.

Dickinson, BL, Clements, JD (1995) Dissociation of *Escherichia coli* heat labile enterotoxin adjuvanticity from ADP-ribosyltransferase activity. *Infection and Immunity* **63**: 1617-1623.

Dickinson, BL, Clements, JD (1996) Use of *Escherichia coli* heat labile toxin as an oral adjuvant. *In:* Kiyono, H, Ogra, PL and McGhee (eds), *Mucosal Vaccines*, Academic Press, pp73-87.

Frame BR, Zhang H, Cocciolone SM, Sidorenko LV, Dietrich CR, Pegg SE, Zhen S, Schnable PS, Wang K (2000) Production of transgenic maize from bombarded type II callus: effect of gold particle size and callus morphology on transformation efficiency. *In Vitro Cellular and Developmental Biolology-Plant* **36**: 21-29.

Gallie, DR, Tanguay, RL, Leathers, V (1995) The tobacco etch viral 5' leader and poly(A) tail are functionally synergistic regulators of translation. *Gene* **165**: 233-238.

Guidry, JJ, Cardenas, L, Cheng, E, Clements, JD, (1997) Role of receptor binding in toxicity, immunogenicity and adjuvanticity of *Escherichia coli* heat labile enterotoxin. *Infection and Immunity* **65**:4943-4950.

Haq, TA, Mason, HS, Clements, JD, Arntzen, CJ (1995) Oral Immunization with a recombinant bacterial antigen produced in transgenic plants. *Science* **268**: 714-716.

Hood EE, Witcher DR, Maddock S, Meyer, T, Baszczynski, C, Bailey, M, Register, J, Marshall, L, Bond, D, Kulisek, E, Kusnadi, A, Evangelista, R, Nokolov, Zivko, Wooge, C, Mehogh, RJ, Hernan, R, Kappel, WK, Ritland, D, Li, CP, Howard, JA (1997). Commercial production of avidin from transgenic maize: characterization of

transformant, production, processing, extraction and purification. *Molecular Breeding* **3**: 291-300.

Kusnadi, AR, Nikolov, ZL, Howard, JA (1997) Production of recombinant proteins in transgenic plants: Practical considerations. *Biotechnology and Bioengineering* **56**: 473-484.

Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**:680-685.

Levine. MM, Kaper, JB, Black, RE, Clements, ML (1993) New knowledge on pathogenesis of bacterial enteric infections as applied to vaccine development. *Microbiology Reviews* **47**: 510-550.

Marks, MD, Lindell, JS, Larkins, BA (1985) Quantitative analysis of the accumulation of zein mRNA during endosperm development. *Journal of Biological Chemistry* **260**: 16445-16450.

Mason, HS, DeWald, DB, Mullet, JE (1993) Identification of a methyl jasmonate – responsive domain in the soybean vspB promoter. *The Plant Cell* **5**: 241-251

Mason, HS, Ball, JM, Shi, JJ, Jiang, X, Estes, MK, Arntzen, CJ (1996) Expression of the Norwalk virus capsid protein in transgenic potato and its oral immunogenicity in mice. *Proceedings of the National Academy of Science USA* **93**: 5335-5340.

Mason, HS, Haq, TA, Clements, JD, Arntzen, CJ (1998) Edible vaccine protects mice against *Escherichia coli* heat labile enteretoxin (LT): potatoes expressing a synthetic LT-B gene. *Vaccine* **16**: 1336-1343.

Millar, DG, Hirst, T, Snider, DP (2001) *Escherichia coli* heat labile enterotoxin B subunit is a more potent mucosal adjuvant than its closely related homologue, the B sub-unit of Cholera toxin. *Infection and Immunity* **69**: 3476-3482.

Moffat, AS (1995) Exploring transgenic plants as a new vaccine source. *Science* **268**: 658-660.

Murray, MG, Thompson, WF (1980) Rapid isolation of high-molecular-weight plant DNA. *Nucleic Acids Research* **8**:4321-4325.

Richardson, SH, Giles, JC, Kriger, KS (1984) Sealed adult mice: a new model for enterotoxin evaluation. *Infection and Immunity* **43**: 482-486.

Sambrook, J, Frittsch, EF, Maniatis, TH (eds) (1989) Molecular Cloning, A Laboratory Manual (2^{nd} ed) Cold Spring Harbor Laboratory Press, Cold Spring Harbor, Chapter 9-31 to 9-62.

Simmons, CP, Ghaem-Magami, M, Petrovska, L, Lopes, L, Chain, BM, Williams, NA, Dougan, G (2001) Immnomodulation using bacterial enterotoxins. *Scandanavian Journal of Immunology* **53**: 218-226.

Snider, DP (1995) The mucosal adjuvant activities of ADP-ribosylating bacterial enterotoxins. *Reviews in Immunology* **15**: 317-348.

Spangler, BD (1992) Structure of Cholera toxin and the related *Escherichia coli* heat-labile enterotoxin. *Microbiological Reviews* **56**:622-647.

Tackaberry, ES, Dudani, AK, Prior, F, Tocchi, M, Sardana, R, Altosaar, I, Ganz, PR (1999) Development of biopharmaceuticals in plants expression systems: cloning, expression

and immunological reactivity of human cytomegalovirus glycoprotein B (UL55) in seeds of transgenic tobacco. *Vaccine* **17**: 3020-3029.

Tacket, CO, Mason, HS, Lasonsky, G, Clements, JD, Levine, MM, Arntzen, CJ (1998) Immunogenicity in humans of a bacterial antigen delivered in a transgenic potato. *Nature Medicine* **4**:607-609.

Yamamoto, T, Tamura, T, Ryoji, M, Kaji, A, Yokota, T, Takano, T (1982) Sequence analysis of the heat-labile enterotoxin subunit B gene originating in human enterotoxigenic *Escherichia coli*. *Journal of Bacteriology* **152**: 506-509.

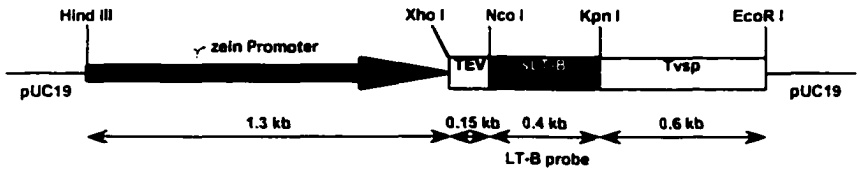


Figure 1. Schematic diagram of construct pRC4 with the synthetic gene encoding the B subunit of LT (sLT-B) under the control of the 27 kD gamma zein promoter, the translational enhancer leader sequence of tobacco etch virus (TEV) and the soybean vegetative storage protein terminator (vsp) in a pUC19 background. The 0.4 kb NcoI – KpnI fragment was used as a probe in Southern analysis.

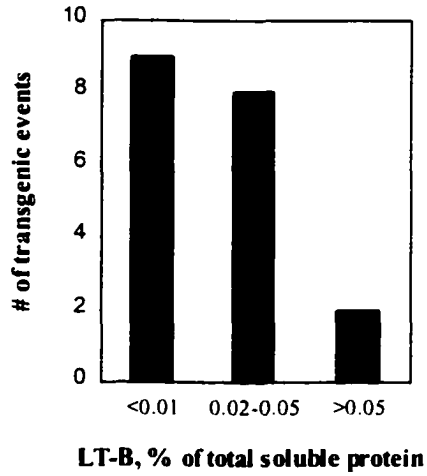


Figure 2. Accumulation of LT-B protein in endosperm of mature seed from nineteen independent P77 R_0 transgenic events. Endosperm tissue was sampled as described in Materials and Methods and the LT-B expression assayed by ELISA. Twenty kernels from two ears per event were analyzed. The LT-B expression level (% of total soluble protein) of each event was determined as the mean of all ELISA positive kernels.

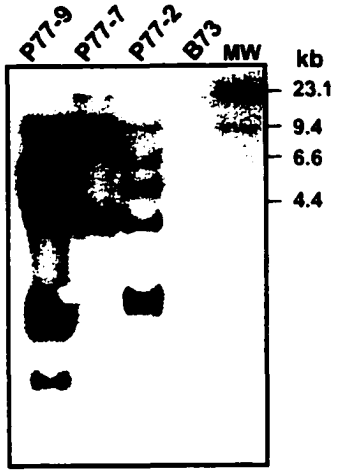


Figure 3. Southern blot analysis of selected transgenic events P77-2, -7 and -9. Ten micrograms of total leaf genomic DNA of R1 plants was digested with NcoI, a restriction enzyme with a single cut within the sLT-B gene cassette (Figure 1). DNA was separated in a 0.8% agarose gel, blotted onto a Zeta-probe nitrocellulose membrane (BioRad) and hybridized with 32P-labeled LT-B gene as probe. MW, Molecular Marker, lambda DNA digested with HindIII. B73, non-transgenic maize DNA as negative control

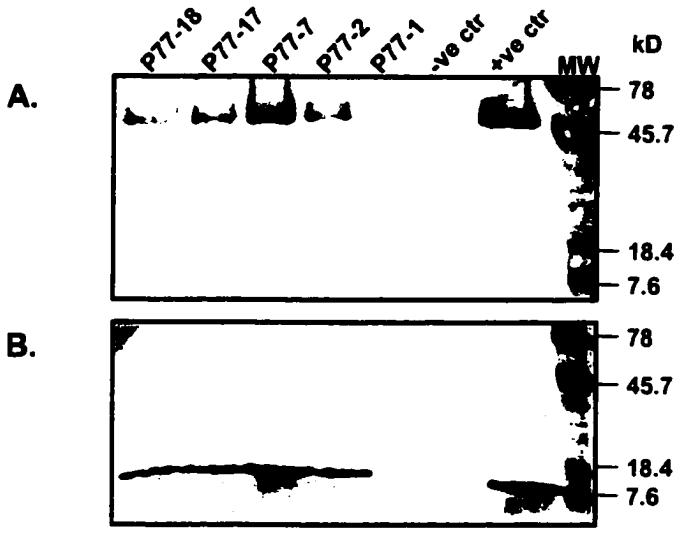


Figure 4. Western analysis of selected transgenic events P77–1, –2, –7, –17 and –18. One hundred micrograms of total soluble protein from R_2 meal was separated on an analytical discontinuous SDS (12%) PAGE, transferred to a 0.45 micron nitrocellulose membrane and probed with goat anti-LT-B and rabbit anti-goat alkaline phosphatase conjugate as primary and secondary antibodies respectively. Panel A, protein samples not boiled prior to loading. Panel B, protein samples boiled (95°C, 5 min) prior to loading. MW, Molecular Weight standards; +ve ctr (100 ng), bacterial LT-B as positive control; -ve ctr, non-transgenic maize extracts as negative control.

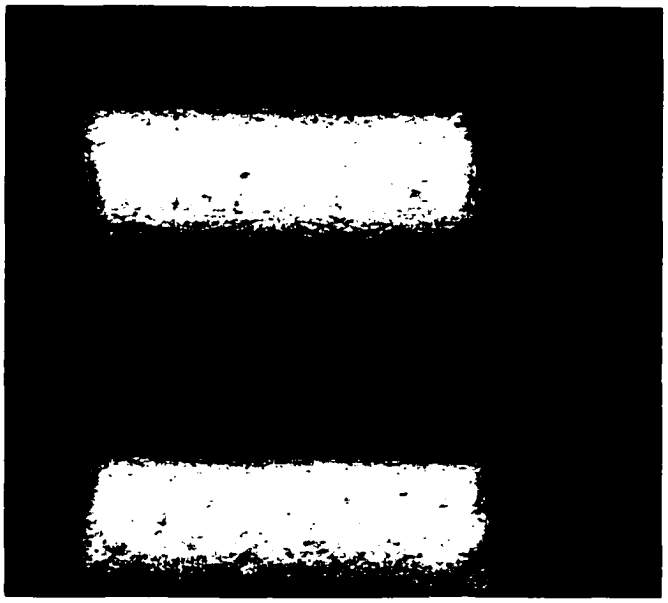
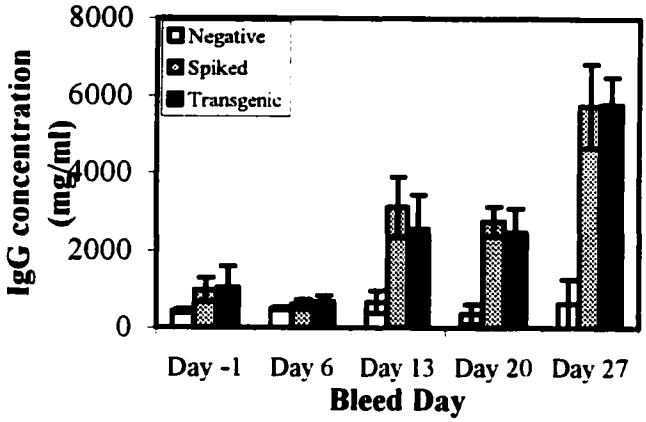


Figure 5. Maize meal pellets used to feed BALB/c mice for oral administration of LT-B. Pellet was made from either non-transgenic maize kernel meal spiked with 10 μg of bacterial LT-B, or transgenic maize kernel expressing 10 μg of LT-B. Each pellet weighed one gram (scale in centimeters).

A.



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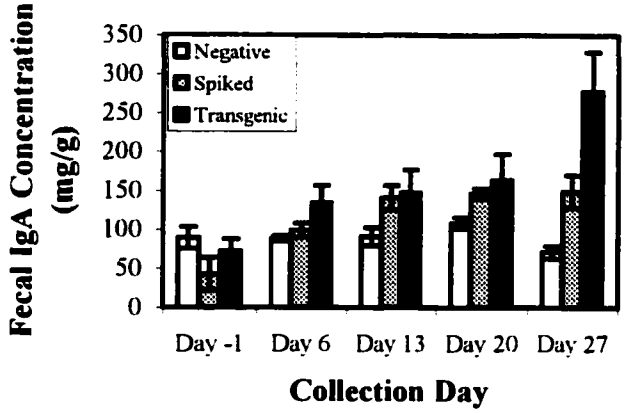

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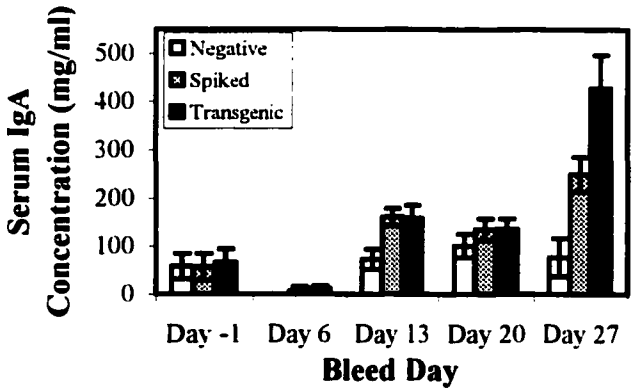
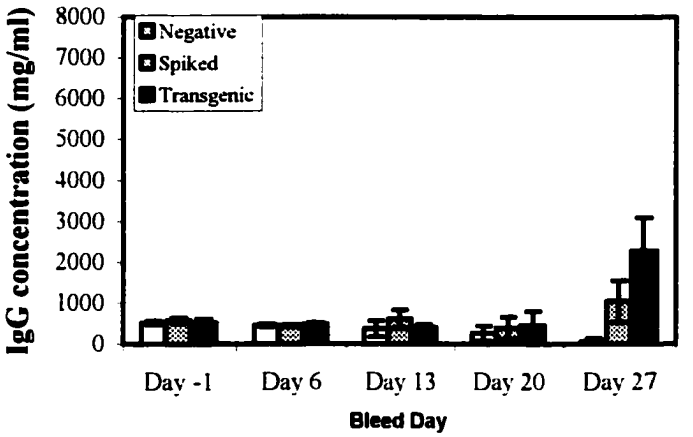


Figure 6. Anti-LT-B antibody analysis in orally immunized mice. Twelve mice in each feed category were fed with maize meal pellets from non-transgenic maize (negative), non-transgenic maize spiked with bacterial LT-B (spiked) and transgenic maize expressing LT-B (transgenic) at days 0, 3, 7 and 21. A. The levels of serum anti-LT-B IgG. B. The levels of fecal anti-LT-B IgA. C. The levels of serum anti-LT-B IgA.

A.



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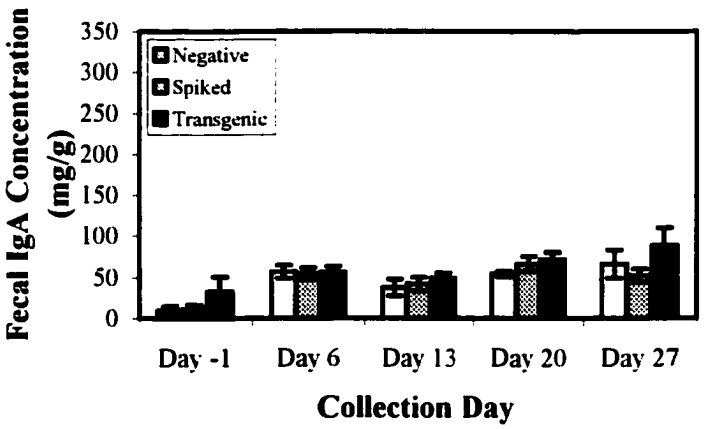

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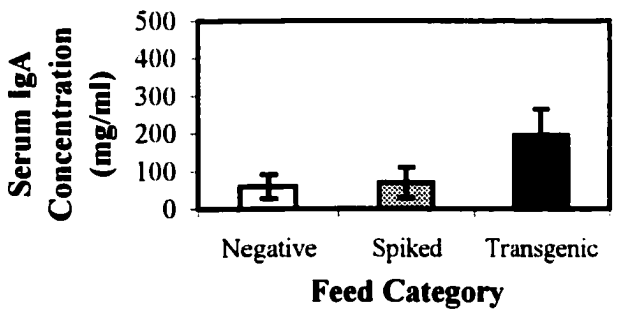
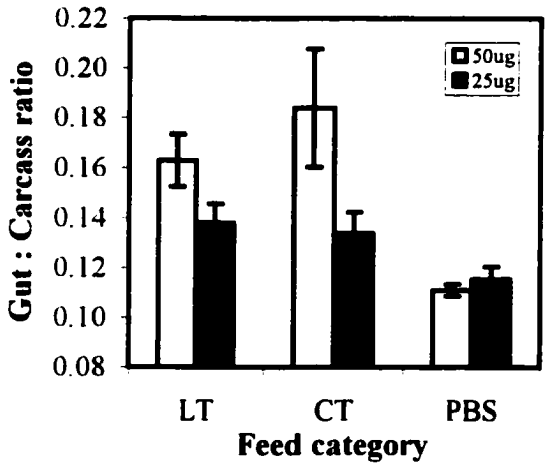


Figure 7. Cross-reacting anti-CT-B antibody analysis in orally immunized mice. Mice were fed with maize meal pellets from non-transgenic maize (negative), non-transgenic maize spiked with bacterial LT-B (spiked) and transgenic maize expressing LT-B (transgenic) at days 0, 3, 7 and 21. A. The levels of serum anti-CT-B IgG. B. The levels of fecal anti-CT-B IgA. C. The levels of serum anti-CT-B IgA at the day 27.

A.



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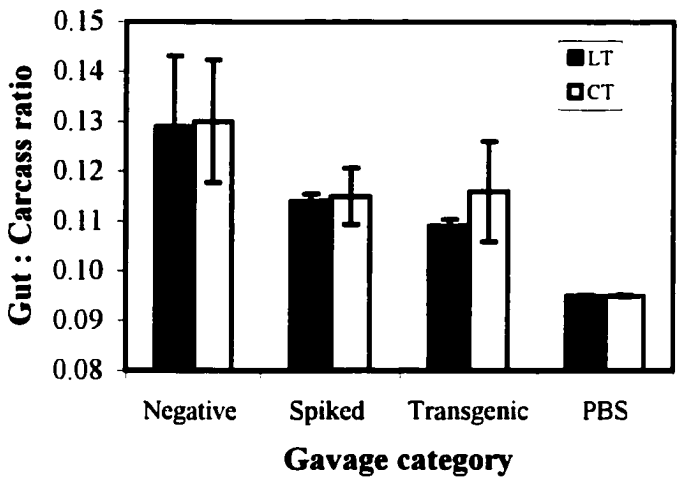


Figure 8. Patent mouse assay for toxin challenge of mice. A. Dose responses of LT, CT and PBS buffer alone. Mice were gavaged with two levels (25 and 50 mg) of LT and CT toxins and dissected 3 hours later to determine the gut/carcass ratio. For each group n=2. B. Response of mice fed with maize meal pellets from non-transgenic maize (negative), non-transgenic maize spiked with bacterial LT-B (spiked) and transgenic maize expressing LT-B (transgenic) to toxin challenge. Each mouse was gavaged with 25 mg of LT or CT or PBS buffer alone at day 27 and gut/carcass ratio was determined 3 hours later as described in Materials and Methods. For each group n=2.

Event ID	Ear ID	Gene copy #	Pollen Source	Seed Yield	LT-B Expression (% TSP)	
					R_1 whole kernels[1]	R_2 meal[2]
P77-1	3-1	nd	Hi II	254	0.01±0.005	0.004
P77-2	2-1	4	selfed	220	0.07±0.007	0.049
	2-2		Hi II	66		0.033
	2-3		selfed	220		0.034
	2-5		selfed	128		0.020
	2-7		Hi II	152		0.047
	2-9		selfed	51		0.036
	2-10		selfed	188		0.022
P77-7	1-1	2	B73	23	0.05±0.002	0.104
	1-2		selfed	97		0.120
	1-3		selfed	76		0.118
	1-4		selfed	154		0.047
P77-9	2-1	5+	selfed	196	0.02±0.002	0.099
	2-2		selfed	53		0.190
	2-6		selfed	35		0.203
P77-17	1-1	nd	B73	23	0.02±0.002	0.008
P77-18	2-1	nd	Selfed	9	0.02±0.005	0.002

[1] Data from 10 expressing kernels from each event.
[2] ELISA was done on pooled kernels from one single ear.

Table 1. Summary of transgene copy numbers, seed yield and LT-B expression of six transgenic events

CHAPTER 5

Generation in transgenic maize calli of a functional non-toxic LT mutant (LTK63) and an LT based candidate subunit vaccines for swine transmissible gastroenteritis virus (TGEV)

Manuscript in preparation.

Abstract

The heat labile toxin (LT) from *Escherichia coli* and its subunits LT-A and LT-B, are potent oral immunogens, which could potentially be used as adjuvants in oral vaccinations with other less immunogenic proteins. LT is more potent as an adjuvant than LT-B alone, but toxicity hampers its use as an adjuvant in oral vaccination programs. This has led to the development of detoxified A subunit LT mutants such as LTK63. We co-expressed LT-B and LT-AK63 genes in transgenic maize callus. Western analysis showed the assembly of LTK63 in transgenic maize callus. The maize generated LTK63 was functional, inducing systemic and mucosal antibodies in orally immunized BALB/c mice. The maize generated LTK63 induced higher serum and mucosal antibody titers than the bacterial LT-B. The expression and proper assembly of functional LTK63 demonstrates that maize can competently produce multimeric proteins that are capable of higher order assembly in transgenic maize tissues. We used LT-AK63 as a carrier molecule for antigens from the spike (S) protein of swine transmissible gastroenteritis virus (TGEV). We fused a 1.12kb N-terminus of the S protein containing critical epitopes for TGEV neutralization to the LT-A

subunit of LTK63. This fusion, LT-A/S, was co-expressed with LT-B in transgenic maize callus under the regulation of the CaMV 35S promoter. We observed assembly of LT-B pentamers and ELISA data suggests possible assembly of these pentamers and the LT-A/S fusion proteins. BALB/c mice orally immunized with 4μg LT-B and LT-A/S fusions induced anti-LT-B antibodies, but no anti-TGEV antibodies could be detected in mice serum.

Introduction

The adjuvant properties of *Escherichia coli* (*E. coli*) heat labile toxin (LT) and its pentameric LT-B subunits have been established. These proteins are potent oral immunogens, which hold great potential in enhancing the value of oral vaccines by eliciting high titer serum and secretory antibodies when administered into the gut (Dickinson and Clements, 1996). Most proteins are not immunogenic when administered orally, and as such have to be either injected, or orally administered along with a strong adjuvant. LT has been shown to be a much more potent adjuvant than the LT-B subunit alone, but the utility of LT as an adjuvant has been hampered by its toxicity. Recent research has focused on the development of non-toxic derivatives of LT (Pizza et al, 2001).

LT consists of five non-covalently bound B sub-units (LT-B) and one A sub-unit (LT-A). The A sub-unit dissociates into two components upon thiol reduction within the cell. The A1 piece has enzymatic activity that causes secretion of water and electrolytes into the lumen of the small intestine, which is characteristic of the debilitating *E. coli* induced watery diarrhea (Spangler et al, 1992). The non-toxic B bind the toxin to the host cell receptor, G_{M1} (galactosyl-N-acetylgalactosaminyl-sialyl galactosyl glucosyl ceramide), which is commonly found on the surface of eukaryotic cells. Interaction of the B subunit

with cell surface receptors triggers the key immunomodulatory events associated with adjuvant activity. LT-B can also serve as an immunologic carrier in traditional hapten-carrier configuration (Cebra et al, 1986). Other antigens conjugated to LT-B can therefore be immobilized for processing by the gut associated lymphoid tissue (GALT).

A number of attempts have been made to alter the toxicity of LT (Dickinson and Clements, 1996). Most of the attempts used site-directed mutagenesis change amino acids associated with the site of enzymatic activity in the A subunit. To date, several mutant LT holotoxins have been developed, including LTK63, LTR72 and LTG192, among others (Pizza et al, 2001). Van den Akker et al, (1997) developed LTK63, a non-toxic mutant of heat labile enterotoxin, by changing Ser63 to Lys through site directed mutagenesis. Pizza et al (2001) showed that LTK63 was a strong and safe oral immunogen upon oral administration. We demonstrate in this work that transgenic maize callus can synthesize from LT subunits and assemble the proteins to a functional LTK63 with immunological activity.

The expression of heterologous antigens as fusion products with the LT-B sub-unit has been described as an optimal strategy for the induction of lactogenic immunity against foreign antigens (Spangler, 1992, Pierce et al, 1980). Antigenic Site D from the transmissible gastroenteritis virus (TGEV) spike (S) protein conjugated to LT-B have been expressed in salmonella and shown to induce virus-neutralizing antibodies in rabbits (Smerdou et al, 1996). Yu and Langridge (2001) demonstrated that an antigen conjugated to the A subunit of CT, a homologue of LT, could be successfully expressed and assembled into a recombinant holotoxin in transgenic potato tissues.

We attempted to exploit the properties of LT and its subunits to develop a maize-based recombinant subunit vaccine against TGEV. TGEV causes a diarrhea disease in pigs, but is especially severe in newborn piglets less than two weeks old where mortality can be up to 100% (Saif and Wesley, 1992). The TGEV S protein is the major inducer of neutralizing antibodies (Garwes et al, 1978, Laude et al 1986). In this protein, four antigenic sites (A, B, C and D) have been defined (Delmas et al, 1986, 1990). Of these antigenic sites, site A is antigenically dominant, sites A and D and to a minor extent site B, have been involved in the neutralization of TGEV (Delmas, 1990). Passive immunity is of primary importance in protecting newborn piglets against TGEV (Saif and Wesley, 1992). This type of immunity can be achieved by the induction of lactogenic immunity in the sow, which in turn can be induced by presentation of selected antigens to the immune system in the gut associated lymphoid tissues (Delmas, 1986).

Attempts have been made to develop a plant-based TGEV edible vaccine by expressing a whole S gene in plants. Welter et al (1996) produced a truncated N terminal domain of the S gene in transgenic potato and Streatfield et al (2001) produced a synthetic version of the whole S gene and expressed it in transgenic maize. These efforts were hampered by low levels of expression of the S protein, although the maize work was promising. We chose an N-terminal domain 1.12 kb common to the S proteins of PRCV and TGEV to be fused to the A subunit of LT. This domain contains the critical antigenic sites (C, D and A) required for TGEV neutralization. The combination of these sites and LT-B with its adjuvant effects constitutes a potentially viable vaccine against transmissible gastroenteritis. The S gene N-terminal domain replaced the A1 subunit in LTK63 in such a way that the assembly of the recombinant A sub-unit to the B5 sub-units would be preserved

(the A2 subunit attached to the S gene fragment), and the B sub-units in the resulting recombinant "holotoxin" could still bind G_{M1} gangliosides.

We observed assembly of LT-B pentamers and ELISA data suggest possible assembly of these pentamers and the LT-A/S fusion protein. Animals were orally immunized with transgenic maize expressing recombinant proteins. Serum and fecal samples were assayed for antibodies against LT-B and TGEV. The callus extract with LT-B and LT-A/S fusions induced anti-LT-B antibodies, but no anti-TGEV antibodies could be detected in mice sera and fecal samples. The mutant LTK63 assembled in transgenic maize calli and induced an immune response in BALB/c mice that was stronger than the response induced by bacterial LT-B.

Material and Methods

Constructs for maize transformation

A fragment encoding the 1.12 kb N-terminal fragment containing the antigenic epitopes A, C and D of S gene was amplified by reverse transcriptase PCR (RT-PCR) from total RNA of the porcine respiratory coronavirus (PRCV). The following primers were used: pLASgen-F1; 5'-cag tgt ccc ggg gat aat ttt cct tgt tcc aat-3'and pLASiteA-R1; 5'-cgt atg acc cgg gag tcc gtg cag ttt cgc tta aaa ac-3'. These primers contained a *Sma* I restriction site to create the necessary blunt ends for ligation into the *Hinc*II sites in the mutated LT-A subunit in pGEM-sLTA-HcHp (Mason, unpublished). The mutated LT-A subunit gene was made by site directed mutagenesis such that A1 sub-unit could be taken out using the engineered *Hinc*II sites, leaving the signal peptide and the A2 domain intact. The N-terminal half of the

S gene generated by RT-PCR was ligated in translational frame with the LT-A bacterial signal peptide and the A2 domain. The S gene fragment fused to the A2 gene was excised from the resultant plasmid using the restriction enzymes *Nco*I and *Sac*I, and used to replace sLT-B in pTH210 (Figure 1A). The resultant plasmid has the S gene fragment fused to the A2 subunit (LT-A/S fusion) under the regulation of the CaMV 35S promoter and the soybean VSP terminator (Figure 1B). The resultant plasmid was called pRC9.

A 2.1 kb fragment from sLTA211 (Mason, unpublished, not shown) including the synthetic LT-AK63 gene, was used to replace the synthetic LT-B in construct pTH210 (Mason et al 1998). The resulting construct pRC9-1 Figure 1C) has the synthetic LT-AK63 gene being regulated by the enhanced CaMV 35S and the soybean vegetative storage protein (VSP) terminator (Mason et al, 1993) from the plasmid. The newly constructed plasmids were sequenced to ensure correct orientation and fidelity of ligation junctions. DNA for maize transformation was obtained using the Qiagen (Qiagen GmbH, Germany) Maxiprep kit according to the manufacturer's instructions.

Maize transformation

Embryogenic maize Hi II callus was transformed using microprojectile bombardment as described by Frame et al (2000). Briefly, the plasmids pRC9 and pRC9-1 were each co-bombarded with the construct pTH210 encoding the synthetic LT-B gene and the selectable marker gene construct, P2. The construct P2 contains an ubiquitin promoter/*bar* gene cassette that confers resistance to the herbicide bialaphos (Frame et al, 2000). Transgenic calli containing plasmid pTH210 encoding the LT-B gene and pRC9 (LT-A/S fusion) and were designated P51P94 and transgenic calli containing pTH210 and pRC9 were designated

P51P95. Herbicide resistant calli were analyzed using the polymerase chain reaction (PCR) for presence of the LT-B, the LT-A/S fusion and the LTAK63 gene cassette.

PCR analysis

Transgenic calli were initially selected on the basis of resistance to bialaphos, the presence of the LT-B, LT-A/S and LTAK63 genes was confirmed by PCR analysis. The transgenes were amplified from 10 to 100 ng of total genomic DNA extracted from 0.1-0.25 g of callus as described by Chikwamba et al, (2002) (Chapter 4 in this dissertation). To amplify the LT-B gene in all transgenic calli, PCR primers and conditions, used as described (Chikwamba et al, 2002, Chapter 4) and were the same in all cases: LTB-PCRF1 (5'-atc gat aca aaa caa acg aat ctc aag c-3') and LTB-PCRR1 (5'-cca tgg cgt gga ttt tat gac att tta t-3'). These 28 bp primers amplify an 842 bp fragment from the TEV leader at the 5' end to part of the VSP terminator at the 3' end, including the LT-B-coding sequence. PCR reactions were carried out in a total volume of 50 µl containing 50-250 ng of maize callus genomic DNA, 5 µl of 10X PCR buffer, 2 µl of dNTP mix (containing 10 mM of each dNTP), 12 µl of each primer (2.5 µM stock), and 1 µl of Biolase Taq Polymerase (Biolase USA Inc., NJ, USA). Reaction conditions were as follows: Initial PCR activation (95°C, 3 minutes) was followed by 30 amplification cycles (denature, 94°C, 30 seconds; annealing, 60°C, 30 seconds, and extension, 72°C, 45 seconds) and a final extension step at 72°C for 5 minutes.

To amplify the LT-A/S gene fusion and the LTA gene, the following primers LTAPCR-F1: 5'-ttc tta taa cag att cag gg-3'and LTAPCR-R1:5'-ttg gta gtc tga gaa gat tt-3'. This primer set amplifies the end of the CaMV 35S promoter through to the end of the LT-A gene, a 1.6 kb and 900 bp fragment in P51P94 and P51P95 respectively. The composition of

PCR mix and PCR cycles were similar to those used for LT-B except that annealing was carried out at 58°C for P51P94, and extension was allowed for 1 minute. The PCR reaction in P51P95 events was performed as described for LT-B.

Protein extraction from callus for ELISA

Evaluation of protein expression was carried out in callus. This procedure was described in Chapter 2 of this dissertation. About 0.25 g of fresh callus was homogenized in 500 µl of protein extraction buffer [25 mM Sodium phosphate (pH 6.6), 100 mM NaCl, 0.5% Triton X-100 (v/v), 10 µg/ml leupeptin (w/v)], after which they were centrifuged at 14,000 rpm in a microcentrifuge for 15 minutes at room temperature. One hundred microliters of the supernatant (50 µl per well, 2 replications/sample) was used for ELISA.

LT-B Expression Assay

For LT-B assay, ganglioside-dependent ELISA was performed as previously described (Mason et al, 1998, Chikwamba et al, 2002) to determine the level of LT-B gene expression.

LTK63 ELISA Assay

To determine the amount of assembled LTK63, ELISA was performed as described for LT-B expression, using rabbit anti-LTK63 polyclonal (Provided by Hugh Mason, Boyce Thompson Institute at Cornell University, Ithaca, New York). Volumes of 50 µl were used throughout all ELISA assays unless otherwise specified. Wells were washed three times between each step using 300 µl of phosphate-buffered saline Tween-20 [PBST; 0.01 M

Na_2HPO_4, 3mM M KH_2PO_4, (pH 7.2), 0.1 M NaCl, 0.05% Tween-20 (v/v)]. Microtiter plates (Costar 3590, Fisher Scientific, PA, USA) were coated with Type III G_{M1} gangliosides (1.5 µg/well) from bovine brain (G2375, Sigma, St Louis, MO, USA), dissolved in sodium carbonate coating buffer (15 mM Na_2CO_3, 35 mM $NaHCO_3$, 3 mM NaN_3, pH9.6) for 1 hour at room temperature. The plates were blocked to prevent nonspecific antibody binding with 5% dry milk (w/v, DIFCO, Becton Dickinson, MD, USA) for one hour at room temperature. Callus extracts were captured on ganglioside coated dishes for one hour at 37°C or 4°C overnight, followed by incubation with rabbit antiserum against LTK63, (1:500 dilution), at 37°C for one hour. This step allowed us to detect any LTAK63 associated with the LT-B and therefore quantify the assembled LTK63. Goat anti-rabbit IgG (KPL) alkaline phosphatase conjugate [1:2500 dilution in 1% dry milk (w/v) in PBS] was added, and the plate incubated for one hour. This was followed by addition of phosphatase substrate, 1 mg/ml ρ-nitrophenyl phosphate (ρNPP, Sigma) in alkaline phosphatase buffer [0.1 M Tris (pH 9.5), 0.05 M $MgCl_2$, 0.1 M NaCl]. Absorbance at 405 nm was immediately measured spectrophotometrically over a two-hour period in a Dynatech MRX Plate Reader (Dynex Technologies, VA, USA). Sample wells were blanked against non-transgenic maize protein extracts, and all measurements were performed in duplicate. Raw ELISA data were converted to LT µg/µl by reference to an ELISA standard curve constructed using purified bacterial LT (kindly provided by John Clements, Tulane University Medical School, LA, USA).

Detection of the S protein fragment

For P51P94, three other ELISA assays were undertaken. To determine the amount of

LT-A/S associated with LT-B, plant extracts were captured on GM_1 gangliosides as described, and reacted with McClurkin swine hyperimmune serum (Kindly provided by Dr Ron Wesley, NADC, Ames, IA, USA) 1:1000 for one hour at 37°C. This was then followed by incubation with rabbit anti-pig horseradish peroxidase conjugate (Sigma, St Louis, MO, USA), at 1:7000 dilution. TMB peroxidase conjugate and peroxidase solution B (KPL, Gaithersburg, Maryland, USA) were mixed and added according to the manufacturer's instructions, and the reaction stopped after five minutes by the addition of 1M phosphoric acid. Absorbance at 450 nm was determined and results presented as absorbance (measured OD) values.

Detection of LT-B associated with the LT-A/S fusion

To determine the amount of LT-B associated with the LT-A/S fusion, microtiter plates were coated with a mixture of monoclonal antibodies 4F6 and 5D5 (Supplied by Dr Ronald Wesley), diluted 1:1000 in coating buffer for 1 hour at room temperature. These monoclonal antibodies are specific to the S gene. The plates were blocked with 5% milk in PBST. Extracts were captured on the monoclonal antibodies for 1 hour at 37°C, and then reacted with goat polyclonal serum against LT-B (Biogenesis), incubated for 1 hour at 37°C. Rabbit anti-goat horseradish peroxidase conjugate [1:7000 dilution in 1% DM (w/v) in PBS] was added, and the dish incubated one hour. This was followed by addition of peroxidase substrate according to the manufacturer's instructions. Absorbance at 450 nm was determined and results presented as absorbance (measured OD) values.

Determination of total protein in callus extracts

Total aqueous extractable protein concentrations in callus extracts were determined by the Bradford Assay (Bradford, 1976) with the BioRad (Hercules, CA, USA) protein dye concentrate using a standard curve derived from bovine serum albumin (BSA) standards.

Immunoblot analysis

A 30-50 µl aliquot containing 100-200 µg of total protein from maize callus was analyzed by SDS-PAGE (Laemmli, 1970). Protein samples were loaded directly on the gel without heat treatment. The separated proteins were transferred to a 0.45 µm nitrocellulose membrane using the BioRad Transblot apparatus according to the manufacturer's instructions. Proteins were evaluated for presence of assembled LTK63 in P51P95 transgenic calli by Western Blot analysis. The analysis used rabbit anti-LT-B antiserum and a biotin conjugated anti-rabbit IgG (Sigma, St Louis, MO, USA) at 1:3,000 and 1:30,000 dilutions, respectively, using the procedures of Blake et al (1984). Identical membranes were also analyzed by rabbit anti-serum against LTK63 and goat anti-rabbit biotin conjugate diluted 1:1500 and 1:30 000 respectively.

To determine molecular weight of LT-A/S fusions, P51P94, extracts blotted onto 0.45 micron nitrocellulose membrane were analyzed with McClurkin hyperimmune serum and anti-pig alkaline phosphatase conjugate (Sigma, St Louis, MO, USA) 1:2500, and 1:10,000 respectively. Identical membranes were also reacted with goat anti-LT-B antiserum and an alkaline phosphatase conjugated anti-goat IgG (KPL) at 1:3,000 and 1:10,000 dilutions, respectively, to determine the position of LT-B relative to the S gene fragments.

Preparation of callus extracts to gavage mice

Fifty grams of fresh actively growing callus were homogenized in a mortar and pestle into a smooth paste. Five milliliters of extraction buffer [25 mM Sodium phosphate (pH 6.6), 100 mM NaCl] without Triton X-100 was added to the pastes and the slurry transferred into 50 ml polycarbonate centrifuge tubes and centrifuged at 11,000 rpm. LT-B protein present in supernatant extracts quantified by ELISA as described. Non-transgenic calli were extracted in a similar manner and used for gavaging the negative control group.

Preparation of viral lysates

TGEV infected swine testicular cell lysates were obtained from Dr Wesley's lab were gamma irradiated to inactivate the virus. Viral lysates were used to gavage mice.

Oral administration of recombinant proteins

Seven-week old female inbred BALB/c mice were obtained from Harlan (Indianapolis, IN, USA) and were allowed a two-week adjustment period with reverse light dark cycle prior to onset of experiment. The mice were kept in the Iowa State University animal facility. All animal procedures were approved by the Iowa State University Laboratory Animal Resources Committee on Animal Care prior to experimentation.

Mice were fasted 12 hours prior to administration of plant extracts or bacterial toxin by gavage with water *ad libitum*. Seven gavage material categories were used for this experiment, as shown in Table 1. These categories were non-transgenic B73 maize callus extracts, LT-B, LTK63, LTB/LT-A/S transgenic callus extracts, irradiated TGEV enriched viral lysates, bacterial LT-B and LT (see Table 1). Mice gavaged with these feed types were

referred to by these categories in the results section. The LT-B, LTK63, bacterial LT-B and LT, were gavaged with 8µg LT-B equivalent in 200µl of plant extract or in the case of bacterial proteins, 8 µg LT-B in 200 µl in non-transgenic callus extracts. The group gavaged with P51P94 callus extracts contained 4 µg LT-B in 200 µl of plant extract. Measurements were based on LT-B levels because LT-B could easily be quantified. Eight mice were used per feed category, except the groups gavaged with enriched viral lysates, and P51P94 callus extracts (Table 1). Two mice were placed in each cage and gavaged with 200 µl of the appropriate callus extracts delivered in a 1 ml syringe attached to standard mice feeding needles. Three hours later and the mice returned to their main cages with their normal rodent food. Mice were fed on days 0, 3, 7, 21, 41 and 53.

Serum and fecal sample preparation

Fecal pellets and blood (approximately 100 µl) were collected once a week prior to fasting and feeding and once before euthanasia (days -1, 6, 13, 20, 41 and 53). Mice were not bled prior to feeding at day 3. Anti-LT-B antibody determination fecal and serum samples were prepared and assayed as described by Chikwamba et al, 2002 (Chapter 4).

Patent mouse assay

The patent mouse assay was also described previously by Chikwamba et al, 2002. The mice were challenged with LT (John Clements) on day 28. The mice were fasted for 12 hours prior to the challenge with water available *ad libitum* and orally gavaged with 200 µl of PBS (pH 7.2) containing 25µg LT. After toxin administration (three hours), the mice were euthanized by carbon dioxide inhalation, weighed, dissected and the gut removed from the duodenum to the anus. The gut, with the fat pad and the mesentery left intact, was weighed

separately. The gut/carcass ratio (Richardson et al, 1984) was then calculated to determine the extent of toxin induced water influx into the gut.

Virus neutralization assay

Three days before the assay, 96-well plates were seeded with ST cells. To check that the virus was still viable, a neutralization assay was carried out using rabbit polyclonal serum against TGEV diluted 1:100. The assay showed that the virus was indeed viable. The test sample sera were heat inactivated at 56°C for 20 minutes before use in test. About 40µl of serum sample from each mouse were used in the assay to start a 1:10 dilution assay. To make serum dilution plates, empty sterile 96-well plates were filled 50µl per well of F-15 with 2% fetal calf serum (FCS), except the first columns, which had 90 µl. Four replications (4X 12 wells) per sample were used in the test. Ten microliters of the first serum were added to the first columns in rows A-D and 10µl of the second serum added to first column in rows E-F for each of the dishes used. Two fold dilutions were made by transferring 50 µl between rows and mixing before transferring to the next row. Twelve dilutions were made, and 50 µl from the last welsl were discarded. Fifty microliters of diluted virus containing 20 p.f.u. in F-15 with 2% FCS were added to each well in the dilution plate, and these plates were incubated at 37°C for 1 hour with CO_2 and humidity. Plates were agitated occasionally. After flicking medium off of the 96 well plate with an ST monolayer, 100 µl from each well of the dilution plate were transferred to the corresponding well on the 96-well plate with an ST monolayer. The virus was incubated on the monolayer for 4 to 7 days, and the titer

checked by microscopy. The final titer was determined fixing cells with methanol and crystal violet staining.

Results

Herbicide resistant transgenic calli were analyzed for the presence of both the LT-B and LT-AK63 or the recombinant LT-A/S genes by PCR analysis. The constructs pRC9 and pRC9-1 were each co-bombarded with pTH210, containing the LT-B gene cassette, and P2 (Frame et al, 2000), encoding bialophos resistance. On average, seventy percent of the herbicide resistant transgenic calli contained all three genes based on herbicide resistance and PCR analysis.

Independent callus events confirmed to be transgenic by PCR were further analyzed by ELISA. Ganglioside dependent ELISA was used to detect and quantify the expression of LT-B in both P51P94 and P51P95 calli. Table 1 summarizes some of the analyses in selected transgenic calli. LT-B expression ranged from 0.005 to 0.07% of total aqueous extractable protein (TAEP) in P51P94 and 0.005 to 0.15% of TAEP in P51P95 events. Some events with substantial LT-B expression did not show a positive PCR reaction or did not have a transcript for the second gene of interest, LT-AK63 (P51P95 Events #3, #26) or the recombinant LT-A/S (P51P94 Events #27, #28).

ELISA assays were also carried out to detect expression of the second gene of interest. For P51P95, extracts captured on gangliosides were reacted with anti-LTK63 polyclonal sera developed in rabbit to detect the assembled LTK63. The rabbit polyclonal serum had a high degree of non-specific reactivity with maize proteins, and showed very high background in non-transgenic maize callus extracts. The antibody reacted poorly with

the normal LT holotoxin that was used as a standard. Events with consistently higher relative absorbance readings than most of the other transgenic and non-transgenic events in this assay were selected for further analysis. RT-PCR and western analysis were used to confirm LTK63 expressing events. The events with higher absorbance in ELISA were associated with the presence of the LTAK63 transcript in RT-PCR analysis. To determine the proportion of the LTAK63 associated with LT-B, western analyses were performed. (Figure 2). Probing with goat polyclonal sera against CT-B showed bands of an apparent molecular weight of about 84 kDa, the size of the assembled native LT. This apparent molecular weight suggests that most of the LT-B and LT-A were associated (Figure 2A), although a minor band of smaller molecular weight (LT-B alone, or LTAK63 associated with fewer than five LT-B monomers) was observed. Similar size bands were obtained when an identical gel was reacted with rabbit polyclonal serum against LTK63 (Data not shown). It is not clear from Western analysis whether the LTK63 was partially assembled or whether it dissociated in SDS-PAGE. Analysis of native LT by SDS-PAGE showed dissociation of the holotoxin into the pentamer and smaller aggregates, suggesting its instability in SDS-PAGE (data not shown). The amount of LTK63 obtained was based on LT-B expression, then estimated by Western analysis (Figure 2). In the case of P51P95 event #30, it was estimated that at least half the LT-B present was associated with LT-A.

For P51P94, four different ELISA analyses were carried out to detect the presence of the recombinant proteins. Level of LT-B expression was detected by ganglioside ELISA as shown in Table 2. In the second ELISA, plant extracts were captured on G_{M1} gangliosides and reacted with anti-TGEV hyperimmune serum to detect the presence of the N-terminus of the S protein associated with the LT-B protein in the plant extracts. It was observed that the

TGEV enriched virus lysates had a high affinity for G_{M1} gangliosides, as did the plant synthesized S protein fragments (data not shown) Events P51P94 #7 and #31 had high relative absorbance readings. Callus extracts from the same events were captured on a mixture of monoclonal antibodies specific to the S protein (4F6 and 5D5), and then reacted with polyclonal anti-TGEV antibodies. Transgenic events with high relative absorbance were observed, including events #7 and #31. Transgenic event P51P95 #9 was used as an internal control to show that the LT-AK63 subunit did not have affinity for anti-S protein antibody.

Extracts expressing recombinant LT-A/S protein were captured by monoclonal antibodies specific for S proteins and reacted with goat polyclonal serum against LT-B to detect the presence of LT-B associated with the LT-A/S recombinant protein. From these analyses, it was shown that events P51P94 #7, 9 and 31 were expressing both the LT-A/S fusion and LT-B proteins. These observations were confirmed by western analysis (Figure 2C and D). These results suggested possible assembly of the LT-B and LT-A/S proteins. Western analysis of P51P94 plant extracts showed the presence of S-protein peptides as well as LT-B expression (Figure 2C and D). Western analysis did not show the presence of high molecular weight products, which would indicate the combination of LT-B and LT-A/S fusions. Another possibility is that assembled recombinant toxin could be dissociated in SDS-PAGE, and would therefore not be observed in Western analysis. While the co-expression of both LT-B and LT-A/S proteins in the same transgenic events were confirmed by western analysis, immunoprecipitation attempts were not successful. P51P94 event #7 was chosen to feed mice because of its substantial LT-B expression, at 0.06% LT-B of TAEP.

Mice feeding experiments were conducted using callus extracts from the transgenic events P51P95 event #30 and P5194 event #7. BALB/c mice were gavaged with homogenate from the transgenic callus expressing the recombinant proteins and bacterial proteins and TGEV infected ST cells as controls. Recombinant and bacterial toxins showed serum and mucosal immune responses (Figures 3 and 4). The results are presented as the mean antibody titer ± standard deviation (SD). Mice gavaged with 8μg of LT had detectable levels of serum IgG at day 13 of the experiment (Figure 3A). The response by the end of the experiment was at 7.2±0.61 mg/ml. The response in mice gavaged with an equivalent amount of plant synthesized LTK63 was clearly distinguishable by 27, and peaked at 5.6±2.2 mg/ml. The response in this group of mice was characterized by higher variability (large SD) compared to the group gavaged with bacterial LT (Figure 3A). This antibody response was higher than that induced by bacterial LT-B (compare 5.6±2.2 mg/ml to 4.1±0.62, Figure 3A). The level of immune response induced by an equivalent amount of bacterial and plant synthesized LT-B was also assayed over the course of the experiment. The plant derived LT-B induced a response that was lower than the bacterial LT-B at 3.1±2.16 mg/ml compared to the bacterial LT-B at 4.1±0.62 mg/ml (Figure 3A). The plant derived LT-B response was also more variable than the bacterial LT-B.

Fecal anti-LT-B IgA was also determined in fecal pellets over the course of the experiment and the results are shown in Figure 3B. Bacterial LT induced 0.090±0.017 mg/ml, while plant LT-derived induced 0.08±0.007-mg/ml anti-LT-B IgA. Plant LT-B induced the lowest anti-LT-B fecal IgA at 0.02±0.008 mg/ml and bacterial LT-B induced 0.06±0.006 mg/ml.

Serum IgA was also measured over the course of the experiment (Figure 3C) to corroborate the observed fecal IgA. The native bacterial LT had a serum IgA titer of 1.7±0.261 mg, compared to plant derived mutant LT (1.5±0.910 mg/ml, Figure 3C). Like the IgG response observed in mice gavaged with plant derived LT had a larger variation in response. Mice gavaged with bacterial LT-B had a higher antibody titer at 0.34±0.012 mg/ml compared to mice gavaged with plant LT-B at 0.2±0.042 mg/ml. Mice gavaged with plant LT-B had a higher level of variation compared to mice gavaged with plant derived LT-B (Figure 3C).

Sera and fecal pellets from mice gavaged with P51P94 extracts were also analyzed for anti-LT-B and anti-TGEV antibodies and the results are presented in Figure 4. Four mice were gavaged with the non-transgenic maize callus extracts (negative group), another group of four mice were gavaged with callus extracts from P51P94 #7, which expressed 4 μg LT-B in 200 μl of extract and S protein peptides (group designated LTB-TGE). The positive control group was gavaged with irradiated TGEV enriched swine testicular cell lysates, and this group was designated TGE. The LTB-TGE group extracts induced serum anti-LT-B IgG (3.11±2.2 mg/ml, Figure 4A) but only background levels of fecal IgA, 0.011±0.008 mg/ml, were observed Figure 4B. The TGE had an unexpectedly high serum anti-LT-B IgG background titer at 1.8±1.66 mg/ml Figure 4A. Serum anti-LT-B IgA levels (0.112±0.04 mg/ml, Figure 4C) confirm the production of IgA antibodies to LT-B, which was observed to be low (Figure 4B). Anti-TGE serum antibodies were not detected in serum from mice in any of these groups.

On day 28, four mice from each of the negative, plant LT, plant LT-B, bacterial LT and bacterial LT groups were subjected to challenge by 25 μg of LT. In each group, two

mice were challenged with 25 μg LT in 200 ml of PBS, and the other two were challenged with 200 μl of PBS. No protection was observed in any of the groups challenged as reflected by the high gut: carcass ratio (data not shown).

Discussion

Synthetic genes encoding *E. coli* heat labile toxin subunits LT-B and the mutant LT-AK63 under the regulation of the constitutive CaMV 35S promoter were co-expressed in transgenic maize callus. ELISA and western analyses showed the presence of the assembled LTK63 holotoxin. An S gene fragment containing the antigenically important A and D epitopes of the S gene derived from PRCV strain was co-expressed with a synthetic LT-B gene in transgenic calli. ELISA and western analysis showed that the N terminal domain fused to LT-A1 subunit was expressed in transgenic maize callus. We demonstrated with this work that maize can competently synthesize a functional LTK63 holotoxin, and a non-optimized fragment of the S gene common to PRCV and TGEV.

The plant optimized LT-B and LTAK63 genes were co-expressed in transgenic maize callus as control for the P51P94 experiment. The two genes had their codon usage (Gribskov et al, 1984) optimized for expression in maize and potato. The two subunits were expressed and assembled into the mutant LTK63 in transgenic maize callus. The level of expression was based on the level of LT-B analysis, because LT-B could be easily quantified by ganglioside dependent ELISA. The rabbit polyclonal antibody against LTK63 could not be used to accurately quantify the amount of LTK63 expressed in maize because of its high degree of non-specific reaction to other proteins in maize (Chikwamba unpublished). Furthermore, in our hands, this antibody reacted poorly with the native bacterial LT that was

used as the positive control and standard. No commercial LTK63 was available for this purpose.

While bacterial LT induced elevated levels of antibodies (IgG and IgA) earlier in the experiment, by the end of the experiment (Day 53), the mutant LTK63 induced antibodies were not significantly different from the antibody titer induced by LT. Furthermore, antibody titers induced by LTK63 were consistently higher than the antibody titer induced by bacteria derived LT-B. The anti-IgA assays showed that the fecal anti-IgA titer was lower than to the serum IgA. The anti-IgA antibody in serum is usually measured to corroborate the observations on fecal IgA assays. These results suggest the possible degradation of antibodies in the fecal pellets. Larger variability in antibody titers was observed in mice gavaged with the plant derived recombinant proteins compared to those gavaged with bacterial LT and LT-B. This variability could be attributed to the stability of recombinant proteins in liquid plant extracts.

The orally immunized mice were challenged with 25 μg of LT at day 27. Results of this challenge show that none of the mice had adequate antibodies to be protected from challenge by 25 μg of LT. The experiment was therefore continued with the remaining mice to monitor the trends in antibody titers. In previous work, oral immunization with maize kernel derived LT-B induced a much higher level of anti-LT-B antibodies by day 27, although higher amount of LT-B was used (10 μg compared to 8 μg in the current experiment). These results suggest that the administration of toxins by gavage may not induce as much anti-LT-B antibody as was observed in previous work. These results indicate that the formulation of plant derived vaccines could have important implications for their effectiveness.

Maize-derived LTK63 induced serum and mucosal antibodies that were higher than bacterial LT-B and comparable to the response induced by the native LT. Mutant LTK63 synthesized in bacteria has been shown to consistently induce serum and systemic antibodies that were higher than those induced by bacterial LT-B (Pizza et al, 2001). However, high variability in the data impedes clear statistical analyses.

We demonstrated that the two genes can be successfully expressed in transgenic maize callus and accumulate in pentameric G_{M1} binding form. The extracts from transgenic calli expressing the mutant LTK63 protein induced anti-LT-B antibodies upon oral immunization of BALB/c mice by gavage. Maize synthesized LT-B induced elevated IgA and IgG serum antibodies and elevated fecal IgA against diarrhea-inducing LT. This work demonstrates that maize synthesized LTK63 is an effective immunogen, inducing in BALB/c mice an antibody titer which is comparable to bacterial derived LT-B and LT. Continuing work in our laboratory involves expression of the mutant LTK63 in transgenic maize kernels under the control of the 27 kDa gamma zein promoter. The LTK63 can be used as an oral vaccine itself or as an adjuvant co-administered with conventional or co-expressed antigens.

ELISA analyses of P51P94 callus were used to detect recombinant proteins and to demonstrate assembly of the LT-B and LT-A/S fusion proteins. Assays in which the plant extracts were captured on a mixture of monoclonal antibodies against the S gene were carried out to determine the presence of S gene peptides in the plant extracts. Absorbance (OD_{450}) readings confirmed the presence of peptides that were recognized by anti-TGEV polyclonal sera, indicating that the transgenic calli were expressing the LT-A/S gene. Because of the lack of purified TGEV virus, the amount of S gene peptides expressed in transgenic calli could not be quantified accurately. ELISA assays in which plant extracts were captured on

G_{M1} gangliosides were used to detect the level of LT-B gene expression and also the amount S protein peptides associated with the LT-B gene, if any. LT-B could be detected in plant extracts captured on anti-S protein monoclonal antibodies, yet bacterial LT-B alone did not bind to these monoclonal antibody. These results suggest assembly of LT-B and the LT-A/S fusion protein. A reciprocal of this experiment (capturing the extracts on G_{M1} antibodies and probing for S gene protein fragments) could not be used to demonstrate assembly of these two proteins because it was shown that the TGEV virus and the plant synthesized S gene fragments had a very strong affinity for G_{M1} gangliosides. This finding was perhaps not surprising because the G_{M1} gangliosides are parts of receptors on epithelial cells in the gut, and some others. The TGEV and PRCV virus make contact with the host through epithelial surface on the respiratory and gastrointestinal mucosa respectively, and G_{M1} gangliosides may play a role in this contact.

The affinity of the S protein for G_{M1} gangliosides could find utility in assays to detect the TGEV and PRCV viruses, and also to capture the virus in anti-TGEV and anti-PRCV antibody determination in various sera using an ELISA method. Assembly of the LT-B and LT-A/S fusions could not be confirmed by immunoprecipitation experiments. This could be attributed to the low LT-B expression in the transgenic events in which strong S gene expression was detected. The rationale for co-expressing LT-B and LT-A/S fusions was that in the scenario that assembly was achieved, the LT-B was expected to deliver the LTA2/S fusion protein to the GALT. Without assembly, it was expected that the LT-B gene in its role as an adjuvant would stimulate the induction of antibodies against the LT-A/S fusion proteins with which it was co-administered. Anti-LT-B antibodies were detected in mice gavaged with plant extracts expressing LT-B and the LT-A/S fusion proteins. However, no

anti-S gene antibodies were observed in mice gavaged with the ST lysates enriched for the TGEV virus or in mice gavaged with plant extracts expressing LT-B and the LT-A/S fusion proteins. There are several possible explanations for this observation. For the plant group gavaged with plant extracts containing LT-B and the LT-A/S fusion proteins, it is possible that the concentration of either LT-B, the S protein or both was not high enough to induce an antibody response by the oral route of administration. Administering the plant extracts parenterally could induce antibodies to prove that the fragment selected had the antigenic epitopes required to induce the virus neutralizing antibodies in mice. As for the group administered with the virus-enriched lysates, it is possible that mouse is not appropriate model for induction of anti-TGEV inducing antibodies by oral administration of the inactivated virus. It is also possible that there was low immune response, which could not be detected by the methods of analyses used. Recovery of transgenic events with high expression of both LT-B and the fusion would be essential to the conclusion of this experiment.

Yu and Langridge (2001) demonstrated that expression of CT-A and CT-B oligomers successfully provides a valuable approach to target plant synthesized antigens to the GALT. These authors fused a 22 amino acid immunodominant epitope of the murine rotavirus enterotoxin NSP4 (66 bp) to the CTB sub-unit and the ETEC fimbrial colonization factor CPA1 (431bp) to the N-terminus of CTA2 sub-unit. We attempted to express a much larger S gene fragment (1.12 kb) in fusion with the LT-A2 subunit. Determining the size limit of fragments that could be fused to these subunits is an area that warrants further investigation.

References

Barackman, J.D., Ott, G., and D.T. O'Hagan. 1999. Intranazal immunization of mice with influenza vaccine in combination with the adjuvant LT-R72 induces potent mucosal and serum immunity which is stronger than that with traditional intramuscular immunization. Infect. Immun. **67(8)**: 4276-9.

Bradford M.M. 1976. A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72**: 248-254.

Cebra, J.J., J.A. Fuhrman, D.A. Lebman, and S.D. London. 1986. Effective gut mucosal stimulation of IgA-committed B-cells by antigen. In Vaccines 86: New approaches to immunization. Developing vaccines against parasitic, bacterial and viral diseases. (Brown, F, Channok, R.M., Lerner, R.A., eds.), pp 129-133. Cold Spring Harbor Laboratory, New York.

Chikwamba R.K., J.Cunnick, D. Hathaway, J. McMurray, H. Mason and K. Wang. 2002. A functional antigen in a practical crop: LT-B producing maize protects mice against *Escherichia coli* heat labile enterotoxin (LT) and Cholera toxin (CT). Transgenic Res. In press.

Delmas, B., M. Gelfi, and Laude, H. 1986. Antigenic structure of transmissible gastroenteritis virus. Domains in the peplomer glycoprotein. J. Gen. Virol. **67:**1405-1418.

Delmas, B., D. Rasschaert, M. Godet, J. Gelfi. and H. Laude. 1990. Four major antigenic sites of the coronavirus transmissible gastroenteritis virus are located on the amino terminal half of the spike glycoprotein S. J. Gen. Virol. **71**: 1313-1323.

Dickinson, B.L., and J.D. Clements. 1996. Use of *Escherichia coli* heat-labile enterotoxin as an oral adjuvant. In Kiyono, H., Ogra, P.L. and McGhee (eds). Mucosal Vaccines. Academic Press. pp73-87.

Frame B.R., Z. Zhang, S.M.Cocciolone, L.V. Sidorenko, C.R. Dietrich, S.E. Pegg, S. Zhen, P.S. Schnable and K Wang. 2000. Production of transgenic maize from bombarded type II callus: Effect of gold particle size and callus morphology on transformation efficiency. In Vitro Cell Dev Biol-Plant **36**: 21-29.

Gallie DR, R.L. Tanguay and V Leathers. 1995. The tobacco etch viral 5' leader and poly(A) tail are functionally synergistic regulators of translation. Gene **165**: 233-238.

Garwes, D.J., M.H. Lucas, D.A. Higgins, B.V. Pike, and S.F. Cartwright. 1978. Antigenicity of structural components from porcine transmissible gastroenteritis virus. Vet. Microbiol. **3**: 179-190.

Gebauer, F., W.A.P. Posthumus, I. Correa, C. Sune, C.M.Sanchez, C. Smerdou, J.A. Lenstra, R. Meloen and L. Enjuanes. 1991. Residues involeved in the formation of the antigenic sites of the S protein of transmissible gastroenteritis coronavirus. Virology **183**: 225-238.

Gribskov M, J. Devereux and R.R. Burgess. 1984. The codon preference plot: Graphic analysis of protein coding sequences and prediction of gene expression. Nucl Acids Res **12**: 539-549.

Laude, H., J.M. Chapsal, J. Gelfi, S. Labiau and J. Grosclaude. 1986. Antigenic structure of transmissible gastroenteritis virus. In: Properties of monoclonal antibodies directed against virion properties. J. Gen. Virol. **67:** 119-130.

Jimenez, G., I. Correa, M.P. Melgosa and L. Enjuanes, L. 1986. Critical Epitopes in porcine transmissible gastroenteritis virus neutralization. J. Virol. **60**: 131-139.

Mason H.S., T.A. Haq, J.D. Clements and C.J. Arntzen. 1998. Edible vaccine protects mice against *Escherichia coli* heat labile enterotoxin (LT): Potatoes expressing a synthetic LT-B gene. Vaccine **16**: 1336-1343.

McKenzie, S.J., and J.F. Halsey, J.F. 1984. Cholera toxin B sub-unit as a carrier protein to stimulate a mucosal immune response. J. Immunol. **133**: 1818-1824.

Park, E.D., J.H. Chang, J.S. Kim, S.I. Chung and J.S. Yum. 1999. Development of two novel nontoxic mutants of Escherichia coli heat labile enterotoxin. Exp. Mol. Med. **31(2):** 101-7.

Partidos, C.D., B.F. Salani, M. Pizza and R. Rappuoli. 1999. Heat labile enterotoxin Escherichia coli and site directed mutant LTK63 enhance the proliferative and cytotoxic T-cell responses to intranasally co-immunized synthetic peptides. Immunol. Letts. **67(3)**: 209-16.

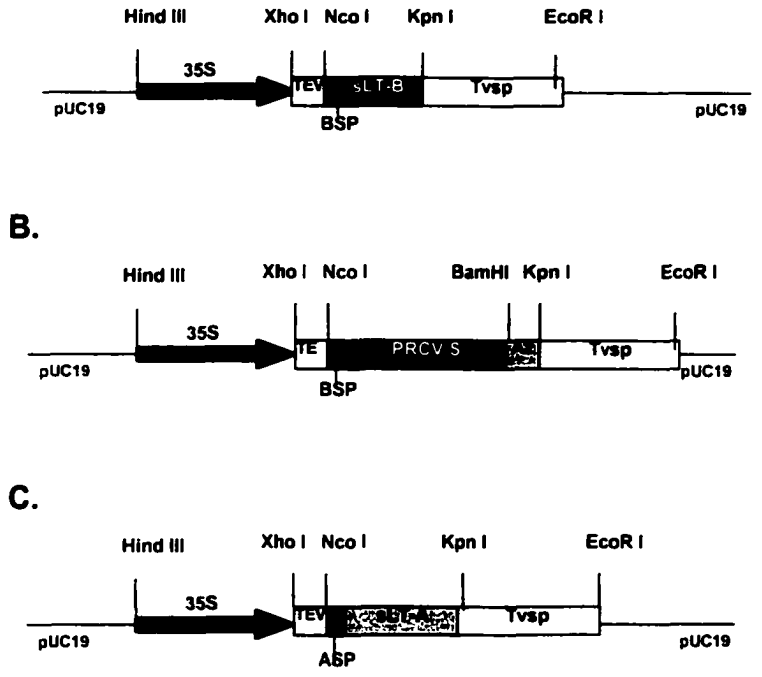
Pierce, N.F., W.C. Craig, and P.F. Engel. 1980. Antitoxic immunity to Cholera in dogs immunized with orally with cholera toxin. Infect. Immun. **27**: 632-637.

Rhee Y. and P.E. Staswick. 1992. Nucleotide sequence for the soybean vegetative storage protein vspB gene. Plant Physiol. **98**: 794-5.

Saif L.J. and R.D. Wesley. 1992. Transmissible Gastroenteritis. In: A.D. Leman, B.E. Straw, W.L. Mengeling, S. D'Allaire and D.J.Taylor (Eds.), Disease of Swine, pp 362-386. Wolfe Publishing Ltd.

Smerdou, C., I.M. Anton, J. Plana, R. Curtiss III and L. Enjuanes. 1996. A continuous epitope from transmissible gastroenteritis virus protein fused to *E. coli* heat labile toxin B subunit expressed by attenuated Salmonella induces serum and secretory immunity.

Spangler, B.D. 1992. Structure of Cholera toxin and the related *Escherichia coli* heat-labile enterotoxin. Microbiol. Rev. **56**: 622-647.



Construct	Co-bombarded with	Transgenic Callus event ID
pTH210	P2	P51
pRC 9	P2, P51	P51P94
pRC9-1	P2, P51	P51P95

Figure 1. Schematic diagram of constructs used for maize callus transformation to generate LT-B-expressing transgenic plants. All constructs are in a pUC19 vector and contain the CaMV 35S promoter, 35S promoter; TEV, tobacco etch virus translational enhancer leader sequence; sLT-B, the synthetic LT-B gene; sLT-A, the synthetic LT-AK63; gene Tvsp, soybean vegetative storage terminator; SEKDEL, endoplasmic reticulum-retention sequence motif; PRCV S, the N-terminal half of the PRCV gene common to TGEV; BSP, bacterial signal peptide from LT-B; ASP, bacterial signal peptide from LT-A.

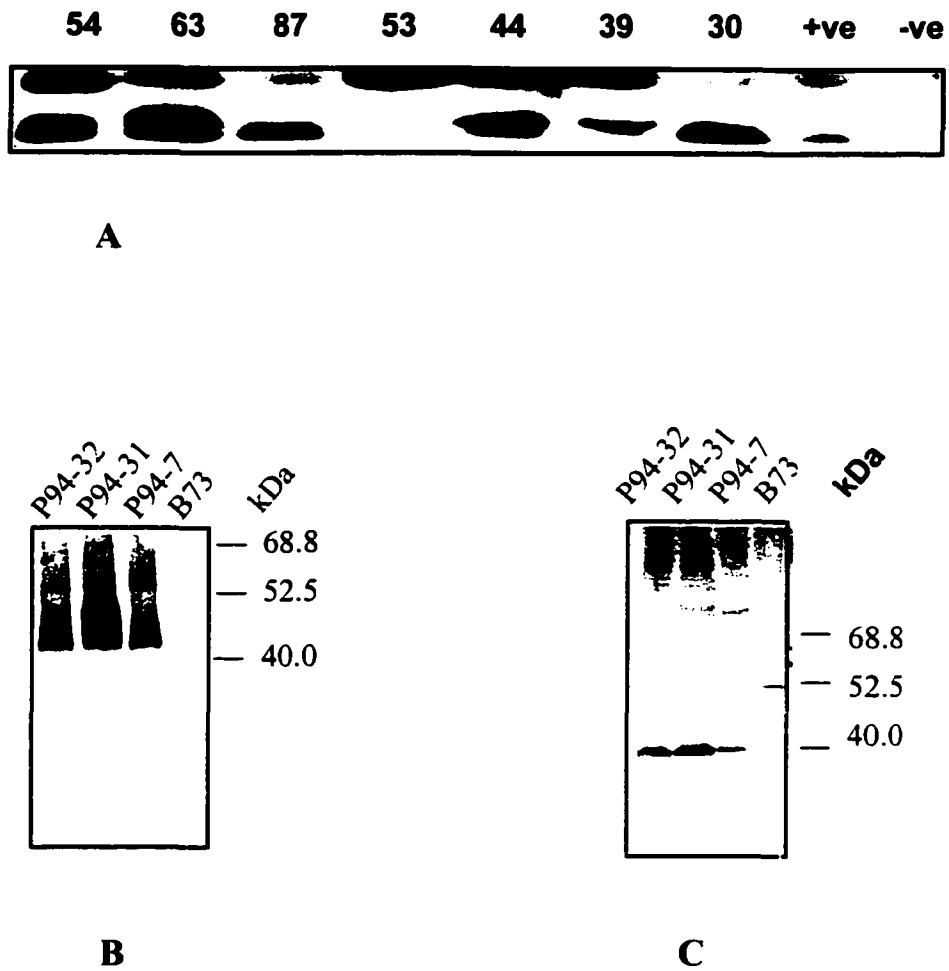
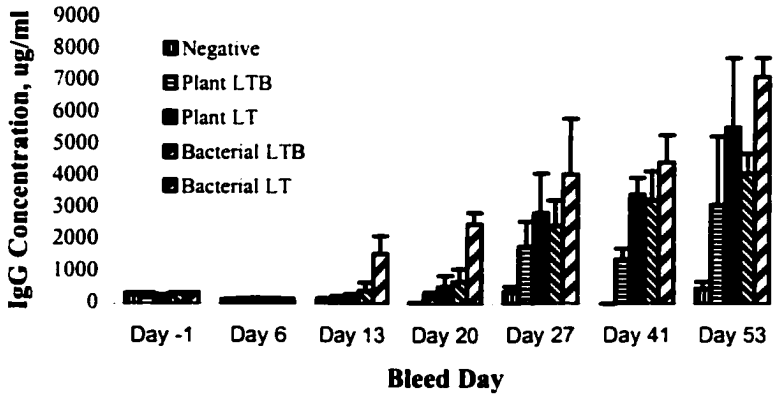


Figure 2. Western blot analyses of total protein extracts of P5194 and P5195 callus. 30-50µl of extracts containing 50-100µg of total protein for P5195 and 100-200µg of total protein for P5194 were loaded onto a 12% SDS-PAGE and transferred to a 0.45 micron nitrocellulose membrane. (A) P5195 extracts probed with anti-CT-B antibodies. (B) P5194 extracts probed with anti-CT-B antibodies. (C) P5194 extracts probed with anti-TGEV antibodies.

A



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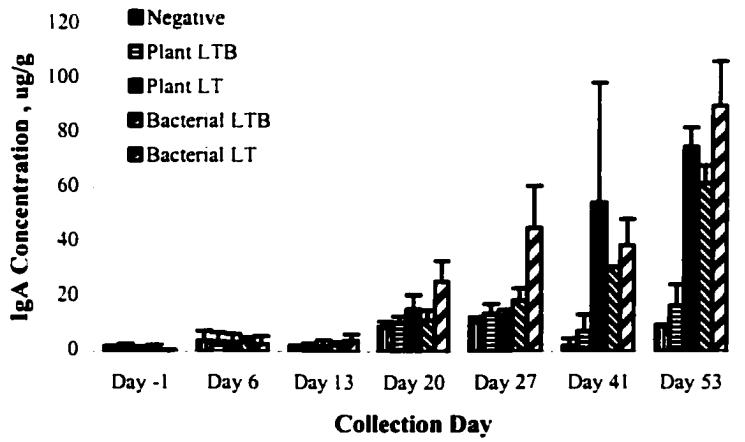

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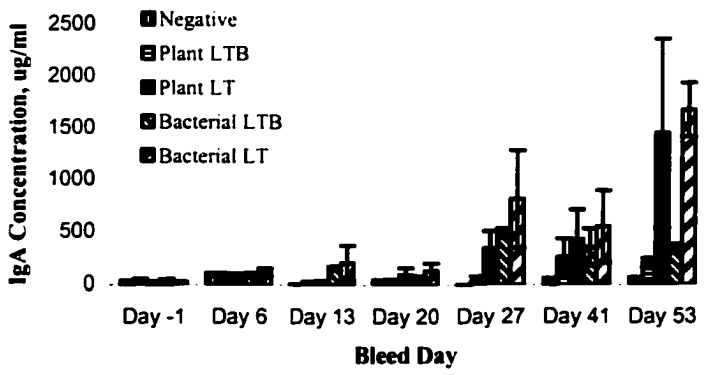
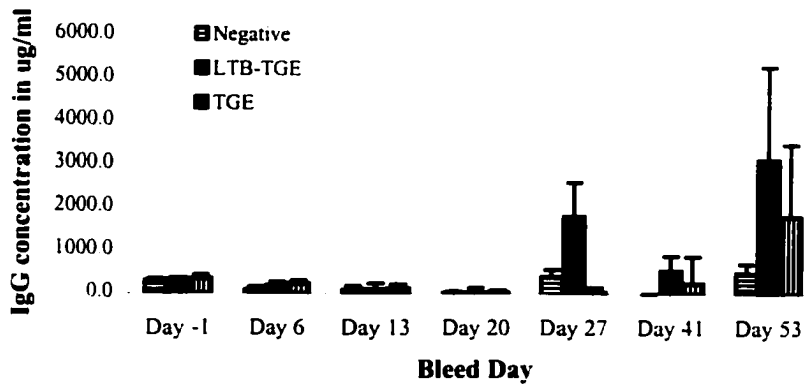


Figure 3. Anti-LT-B antibody analysis in orally immunized mice. Mice in each feed category were gavaged with nontransgenic maize callus extracts (negative), plant derived LT-B from P51 calli (plant LT-B), plant derived LTK63 from P5195 calli (plant LT), bacterial LT-B and bacterial LT on days 0, 3, 7 and 21, 41 and 53. A. The levels of serum anti-LT-B IgG. The levels of fecal anti-LT-B IgA. The levels of serum anti-LT-B IgA.

A



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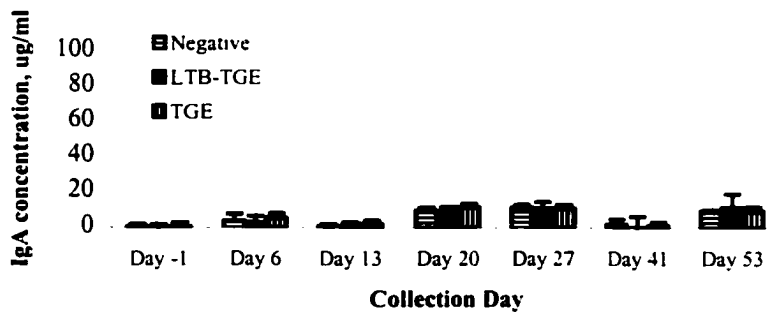

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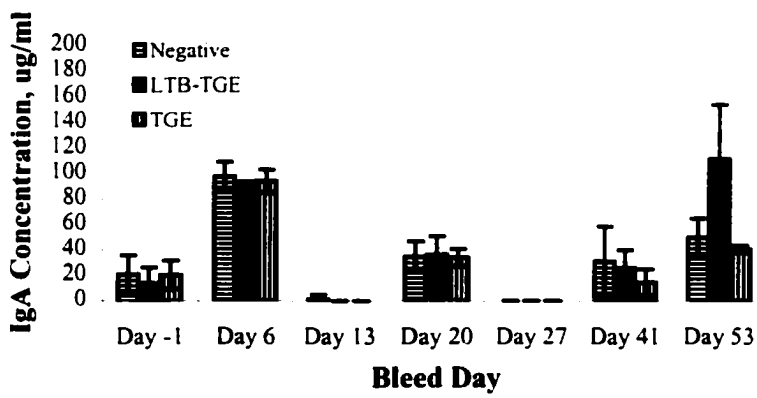

Figure 4. Anti-LT-B antibody analysis in orally immunized mice. Mice in each feed category were gavaged with non-ransgenic maize callus extracts (negative), plant derived LT-B and LTA/S fusion from P51P94 calli (LTB-TGE) and TGEV enriched ST cell lysates (TGE), on days 0, 3, 7 and 21, 41 and 53. The levels of serum anti-LT-B IgG. The levels of fecal anti-LT-B IgA. The levels of serum anti-LT-B IgA.

Construct/ Genotype	Proteins	# of mice assigned	Group Designation
B73	none	8	Negative
P51	Plant synthesized LT-B	8	Plant LT-B
P5195	Plant synthesized LTK63	8	Plant LT
N/A	Bacterial LT-B	8	Bacterial LT-B
N/A	Bacterial LT	8	Bacterial LT
p5194	Plant LT-B/LTA/S	4	LTB-TGE
N/A	Virus enriched lysates	4	TGE

N/A indicates categories that did not derive from plant extracts

Table 1. Categories of material gavaged and number of mice assigned to each category and group reference for result description

Construct	Event ID	LT-B PCR (+/-)	P94 or P95 PCR (+/-)	% LT-B in TAEP	Northern transcript transcript P94 or P95 (Y/N)
P51P94	3	+	+	0.005	+
	4	+	+	0.03	+
	5	+	+	0.01	nt
	7	+	+	0.06	+
	9	+	+	0.01	+
	10	+	+	0.02	nt
	13	+	+	0.01	+
	15	+	+	0.04	+
	19	+	+	0.02	-
	20	+	+	0.03	+
	25	+	+	0.01	-
	26	+	+	0.03	+
	27	+	-	0.07	-
	28	+	-	0.04	-
	29	+	+	0.005	nt
	30	+	-	0.04	-
	31	+	+	0.005	+
	32	+	+	0.01	+
P51P95	1	+	-	0.005	nt
	2	+	+	0.06	nt
	3	+	-	0.03	-
	4	+	+	0.005	nt
	9	+	+	0.04	+
	11	+	+	0.005	+
	12	+	+	0.005	-
	13	+	+	0.005	-
	15	+	+	0.01	-
	16	+	+	0.005	+
	17	+	+	0.01	+
	19	+	+	0.005	+
	20	+	+	0.005	+
	21	+	+	0.005	-
	22	+	+	0.01	-
	26	+	+	0.15	-
	27	+	+	0.01	-
	28	+	+	0.05	+
	30	+	+	0.11	+

nt indicates events that were not tested in RT-PCR

Table 2. Analysis of selected P94 and P95 transgenic events. LT-B levels expressed as % LT-B in total aqueous extractable protein.

CHAPTER 6.

Comparison of antigenic and evolutionary relationships among some Coronaviruses: implications for the elucidation of structure function relationships and development of oral vaccines.

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Manuscript in preparation

Summary

The S glycoprotein is the structural protein of the large, petal-shaped spikes which are characteristic of the coronaviruses, and plays a major role in critical processes such as host cell receptor binding, induction of fusion of viral envelope with cell membrane, induction of neutralizing antibodies and eliciting cell-mediated immunity. Protein sequences of the spike (S) protein of 12 coronaviruses were collected from GenBank, aligned and subjected to phylogenetic analysis. The analysis of the sequence data produced dendograms, which supported the traditional groupings based on serological evidence. The high confidence level along the tree as reflected by bootstrap values suggests the inferred evolutionary history is reliable. While the relatedness of these viruses supports their classification into serological subgroups, specific genomic sequences are yet to be directly associated with specific functions such as virion binding, alterations in biological activity, or even tissue tropisms, and virulence. Regions of high and low sequence homology suggest that regions which code for protein domains that define cross-reactive group epitopes and virus specific epitopes may be highly conserved. As the crystal structure of the S protein has not yet been resolved, it is difficult to assess the significance of non-conserved regions.

Epitopes inducing virus-neutralizing antibodies have been mapped to the S protein of several coronaviruses. We examined the conservation of epitopes and other regions of known function across the S genes in the different coronaviruses with reference to the coronavirus TGEV. The result of this analysis was that critical epitopes are highly conserved within the antigenic clusters. Regions thought to be involved in the conformation of the peplomer protein are also highly conserved in all the sequences analyzed. Viruses evolve rapidly, yet certain key sequences have remained highly conserved. This paper examined how the sequences have changed within antigenic clusters and from one antigenic cluster or evolutionary clade to the next. Some potentially function-determining changes were observed that we speculate to be major contributors to the antigenic cluster. Although sequence alignment is not commonly used for elucidation of structure function relationships in viruses, it is becoming increasingly common in fields such as bioinformatics. Ultimately sequence alignment could contribute to the resolution of the viral protein three-dimensional structure.

Introduction

Coronaviruses are a genus in the family *Coronaviridae*. They are large, enveloped, positive strand RNA viruses and are important pathogens in humans and domestic animals. They are noted as having the largest genomes of all RNA viruses and replicate by a unique mechanism, which results in a high frequency of recombination (Holmes and Lai, 1996). The unique replication strategy involves discontinuous RNA synthesis and polymerase jumping, and can result in recombination rates of up to 25% (Baric et al, 1974). Coronaviruses were initially recognized as a separate virus group by their distinctive virion

morphology in negatively stained preparations, (MacIntosh, 1974) (Figure 1). The viral envelopes are studded with long, petal-shaped spikes, and the nucleocapsids are long flexible helices. Typically, coronaviruses are characterized by a restricted host range and tissue tropism. Other classifying features include their replication strategy, genome organization and mRNA structure, nucleotide sequence homology, serology and properties of other structural proteins.

Serological classification

Coronaviruses have been grouped into three serologically distinct groups on the basis of antigenic relationships (Table 1). Within each sero-group, their natural hosts and antigenic cross-reactivity identify viruses (Sanchez et al, 1990). Viruses within an antigenic group can be distinguished from one another by virus neutralization titers or hemagglutination inhibition tests when homologous and heterologous antibody titers are compared (Gerna et al, 1985). Most coronaviruses naturally infect only one species or several closely related species. Virus replication *in vivo* can cause systemic infections or, localized infections if restricted to a few cell types, usually the epithelial cells of the respiratory or enteric tracts and macrophages.

Coronavirus structural proteins

The genomic organization and RNA sequences of many coronaviruses have now been determined. Coronavirus viral RNA codes for three types of protein the nucleocapsid (N) phosphoprotein, the membrane (M) and the spike (S) or peplomer protein. Exceptions to this include the human coronaviruses of the OC43 group, bovine coronavirus (BCV), porcine

hemagglutinating encephalomyelitis (HEV), turkey coronavirus (TCV), which possess an additional surface glycoprotein, the haemagglutinin-esterase (HE) (Hogue and Brian, 1986, Holmes and Lai, 1996). The M, N and S viral proteins are all important for inducing the immune response to coronaviruses (Buchmeir et al 1984, Delmas et al, 1990) but studies of the induction of protective immunity have focused on the S gene as the major inducer of neutralizing antibodies. The S glycoprotein is the structural protein of the large, petal-shaped spikes (peplomer) (Figure 1). Each peplomer consists of a dimer or trimer of S proteins (Cavanagh, 1983). In coronaviruses such as MHV and infectious bronchitis virus (IBV) but not FIPV or TGEV, the S glycoprotein is cleaved into two non-identical sub-units, amino-terminal S1 and carboxy-terminal S2, probably containing the global and stem portions of the molecule respectively (Spaan et al 1988). The precise residue where the stem part of the S peplomer might start could be revealed by the elucidation of the S protein's atomic structure.

The deduced amino acid sequences of many coronavirus S genes shows many potential N-linked glycosylation sites and 4 structural domains which include a short carboxy-terminal cytoplasmic domain, a transmembrane domain and two large external domains called S1 and S2 (Wesseling et al, 1994, Holmes and Lai, 1996). The cytoplasmic domain is rich in cysteine residues, which could be part of a complex tertiary structure involved in the assembly of the spikes or in interaction with other viral proteins (Holmes and Lai, 1996). The lengths and nucleotide sequences of the S1 glycoproteins of different coronaviruses are quite diverse and such variation has been observed even in different strains of a single coronavirus. Holmes and Lai (1996) suggest that the diversity in S1 probably results from mutation and recombination between coronaviruses and strong positive selection *in vivo*, and such changes in S1 have been associated with altered antigenicity and virulence.

However, several hypervariable regions in the S1 sequence where large deletions or insertions commonly occur may represent externally oriented loops that are not essential for the structure of spikes (Paul et al, 1994).

The S protein has many important biological functions (Paul *et al.* 1994), including binding to specific host cell receptor glycoprotein, induction of fusion of viral envelope with cell membrane,and induction of neutralizing antibodies and eliciting cell-mediated immunity. Of interest to this discussion, is the fact that monoclonal antibodies to S can neutralize virus infectivity and inhibit membrane fusion, effectively curtailing viral replication in host cells.

Recently there has been a surge in the desire to develop recombinant oral vaccines against coronaviruses by expressing antigenic proteins in baculovirus and adenovirus (Callebautt et al, 1996, Torres et al, 1995, Shoup et al 1997 and Tuboly et al 1994). Oral vaccines can be developed through the expression of immunogenic viral epitopes in a viral expression vector or in edible parts of crops that are used as feed. Expression of proteins in baculovirus allows for production of foreign proteins in high amounts and allows for post-transcriptional glycosylation where necessary (Shoup et al 1997). Considerable effort is being made toward the development of plant based oral vaccines (Welter et al, 1996, Mason et al 1998). One of the critical aspects of such initiatives is to decide which antigenic epitopes to express in the vaccine delivery system. The S gene has been the candidate antigen in most cases. In this paper we examined the conservation of epitopes across the S genes in the different coronaviruses. Conservation of epitopes across those viruses that affect gastrointestinal and respiratory tracts has practical implications in the development of oral vaccines. Shoup and coworkers 1997 showed the immunogenicity of various fragments of the S protein expressed in baculovirus and their potential to neutralize the swine TGEV.

Human trials of an oral vaccine against traveler's disease, a bacterial disease that causes diarrhea in humans (Mason et al. 1998) indicate that plant based oral vaccine have a lot of potential as effective vaccines against pathogens. Expression of animal viral proteins in plants may necessitate a redesign of the immunogenic protein gene to optimize codon use and enhance expression. This wholesale codon optimization requires redesigning the gene in question so usually the relevant epitope(s) is chosen for expression because it is usually smaller and easier to redesign. It would be prudent to select an antigenic site, which produces virus-neutralizing antibodies against a virus as well as other closely related viruses.

This paper aims to examine the evolutionary relationships among coronaviruses by comparing the S genes. Because of the key role of the S glycoprotein in immunogenicity in coronaviruses, considerable effort has been made to study the structure and functional importance of the various domains in different coronaviruses. While immunogenic epitopes have been identified on the S protein for the different viruses, specific genomic sequences have yet to be directly associated with alterations in the biological activities in the coronaviruses. We expect that regions, which code for protein domains that define cross-reactive group epitopes and virus specific epitopes to be highly conserved. The S gene of the swine transmissible gastroenteritis virus (TGEV) has been extensively characterized, and so for the most part, will be used as a reference.

We have made a simple analysis of evolutionary relationships among several members of the Coronaviridae based on deduced amino acid sequences for the S gene to determine if they reflect the serological sub-groupings into which the family members have been classified. Aligning the sequences has permitted examination of just how conserved certain key features of the protein are across the different groups, to see how they correlate

with function (e.g. tropisms). It is expected that this type of analysis will shed light on structure function relationships. The results of our analyses of relatedness of S sequences support the classification of the viruses into their serological sub-groups. This reinforces the importance of this protein as a potential candidate for the development of oral vaccines.

Materials and Methods

Twelve coronavirus S protein gene sequences were collected from the GenBank database on the World Wide Web and they included the S genes from porcine transmissible gastroenteritis virus (TGEV), porcine respiratory coronavirus (PRCV), murine hepatitis virus (MHV), feline infectious peritonitis virus (FIPV), feline enteric coronavirus (FECV), human respiratory coronavirus (HCV strains 229E and OC43), bovine coronavirus (BCV), canine coronavirus (CCV strains Insavc and K378), avian infectious bronchitis virus (IBV) Beaudette strain and another novel avian IBV strain (designated avian). There were several viruses shown in the antigenic clusters in Table 1, for which sequences were not readily available on the public databases. These DNA sequences were converted to amino acid sequences using the computer program Se-AL (version 1.0 alpha1, 1996). The sequences were aligned using the computer program Clustal X, and analyzed using the programs PAUP (Version 4.0), for construction of evolutionary trees and in McClade (Version 3.07) to determine character states, conservation of domains and other features.

Results and Discussion

Evolutionary relationships and antigenic clusters

The clades on the evolutionary tree shown in Figure 2 reflect the antigenic clusters shown in Table 1. The bootstrap support values (Felsenstein, 1985) shown in (Figure 2) are very high, with a consistency index of 0.97 reflects the strength of the evolutionary relationships among these groups. The same tree topology resulted for neighbor joining, UPGMA and parsimony. The concordance between antigenic classification and the clades on the evolutionary tree might be expected as the evolutionary tree was determined on the basis of homology in the S protein, which, as mentioned earlier, is the main site of several immunogenic motifs. The fact that the evolutionary relationships developed from the S gene show such unambiguous support as reflected by bootstrap suggests the relationships between these viruses are correct. It also implies that the S gene is the major site of evolutionary change that has led to, or at least contributed significantly to the appearance of new strains. The S gene is the site of viral binding to host cells and other critical regions. In this case, HCV-229E was used to define the out-group on the basis of its nucleotide distinctness from the rest of the coronaviruses, including HCV-OC43, another human coronavirus.

Comparison amino acid profiles

An analysis was made of the general conservation of amino acids across the protein for all 12 sequences in the data set using McClade (Version 3.07, 1997). This profile shows that generally residues are quite variable across the proteins, although some regions are obviously more variable than others are. However, when the data is split into the two major

clades on the evolutionary tree, very striking differences are observed (Figures 3). The antigenic cluster I group show marked conservation in the N-terminus, as shown in figure 3(a). According to the literature, the critical antigenic motifs for sero-group I are present as small highly conserved sites in the N-terminus, which for the most part is highly variable (Delmas *et al,* 1986). Delmas and others (1990) propose that the high level of cross neutralization in this group is due to a clustering of most of the critical neutralization epitopes within the structurally conserved and immunodominant N-terminal 26K region. The antigenic clusters II and III combined on the other hand, show conservation in the C-terminus as reflected by figure 3(b). It therefore seems that the antigenic determinants for these two groups are found on opposite ends of the S protein.

Analysis was also made of the frequency of amino acid residues. Hydrophobic amino acids such as leucine are most prevalent. Other prevalent hydrophobic amino acids such as valine, serine and threonine are also quite abundant. While the specific amino acids are variable, the hydrophobic profiles of these proteins are well conserved. Figure 4 shows bubble plots showing the relative frequency of amino acid changes counted over the inferred evolutionary tree, split along the antigenic clusters I and II and III combined. Most of the prevalent changes are between the hydrophobic amino acids, which effectively does not change protein function. Changes like this include those from leucine to phenylalanine and from valine to isoleucine. This is consistent with the generally hydrophobic nature of this protein and can perhaps be explained by the fact that the S protein has several hydrophobic domains. These include a trans-membrane domain and a second strongly hydrophobic domain in the carboxy-terminus, the trans-membrane anchor (Figure 6(a)) (Rasscheaert and Laude, 1987). Another contributor to the protein's hydrophobicity is 14-19 amino acid

stretch at the amino terminus of the S protein, which comprise the signal peptide. What is most striking is the difference in the two profiles of unambiguous changes for the two main subgroups (Figure 4). While the changes within the hydrophobic group are most prevalent, cluster I seems to have fewer changes than clusters II and II combined.

Changes within antigenic clusters

Substitution of hydrophilic for hydrophobic amino acids is of particular interest as this may relate to differences in function (virulence, tropism etc). An analysis between particular strains, which highlights such changes, would be particularly useful. These changes would be especially useful in regions whose functions are known. For example, in some proteins, regions with enzymatic action can barely tolerate such changes. We examined the alignments for such functionally important changes between and within antigenic clusters. There are several regions that have many successive changes as the clades branch off, some of them are conserved within the clades and some are not. It is not easy to discern the importance of such changes, especially when they occur without some obvious or systematic pattern, although it can be concluded that the conserved ones must be of some functional importance. Where cluster I branches of, there is a region of rapid change that is observed from residues 560-583. These changes are occurring within the immunodominant site A, which is highly conserved in this cluster. In view of the antigenic importance of this site, these could be part of the defining changes for this cluster. Changes at residues 560 (Ser-Met), 563 (Val-Ser) and 566 (Lys-Gly) occur with such regularity they display the periodicity of a helix, a feature that is important in functional regions, which this region is.

Analysis of regions of known functions

Several alignments were made after with different values for a gap penalty and the antigenic clusters are consistently maintained to reflect the strength of these relationships. Differences in variability profiles can be used to identify shifts in function at molecular level. On the other hand, regions that are structurally important are more tightly constrained therefore less free to vary. Sequence comparisons can also shed light on functionally important candidate sites for experimental verification using site directed mutagenesis, circumventing the need for random mutagenesis (Naylor et al, 2000). Variations in regions in which some function is known were examined and are discussed individually below.

Signal peptides

There is a 14-19 amino acid stretch of hydrophobic amino acids at the beginning of each protein, which contribute to the overall hydrophobicity of the S glycoprotein. This hydrophobic signal peptide is required to ensure that the S protein attached to the endoplasmic reticulum where the virions are assembled. The signal peptides of viruses in each antigenic cluster consistently align.

Antigenic Epitopes

The S gene is the site of critical immunogenic epitopes, which induce virus-neutralizing antibodies in the host. In the swine transmissible gastroenteritis virus, the critical antigenic epitopes have been mapped to the N-terminal half of the S gene. Immunogenic epitopes (A-D) have been mapped in the S gene (see cartoon in Figure 5). Sites A and D (Figures 6(a) and (b) respectively) are fairly well defined and have been

demonstrated to be capable of inducing virus neutralizing antibodies the host system. Site A is the immunodominant epitope and is necessary for induction of virus neutralizing antibodies *in vivo*. This epitope is thought to be conformation and glycosylation dependent and in TGEV this stretches from residues 538-591. Site D (figure (6b)) on the other hand is thought to be continuous and linear, in TGEV stretches from residues 378-395 and includes the critical residues **SSFSYGEI**. Mutations in site D in TGEV were noted to lead to markedly reduced pathogenicity. Sites A and D are highly conserved in the first antigenic cluster, and both have hydrophilic residues as the critical residues, indicating that they are most likely exposed on the surface of the spike protein to interact with the host cells. In the different alignments that were made, these residues consistently align, and corresponding positions in sequences that are not in the same antigenic cluster are noted to have neither the same nor similar hydrophilic residues.

Site B in TGEV stretches from residues 97-144, this sequence is only partly conserved in other viruses in this cluster. Site C on the other hand is poorly defined. and in PRCV, a mutant strain of TGEV, these sites are missing. TGEV and PRCV are very closely related and deserve special mention. Nucleotide sequences of PRCV have a characteristic deletion in the 5" end of the S gene compared to TGEV. TGEV causes severe diarrhea with high mortality in neonatal swine. It replicates in and destroys the enterocytes of the villus epithelium in the small intestine, which causes the subsequent malabsortion and dehydration characteristic of transmissible gastroenteritis. TGEV has also been shown to replicate to a limited extend in the respiratory tissue of infected swine. PRCV is believed to be a mutant of TGEV, as it has been shown to be antigenically and genetically related to TGEV but has a selective tropism for respiratory tissue with very little or no replication in the intestinal tissue

of infected swine. Since TGEV and PRCV are closely related and yet display differences in tissue tropism and pathogenicity, they serve as useful models for the study of coronavirus genes involved in tropism and virulence (Vaughn et al 1995).

Receptor Binding sites

Sanchez and others (1990) suggest that tissue tropism of coronaviruses is conditioned by the S glycoprotein and the respiratory or enteric tissues could recognize receptor-binding sites in the S proteins. Virus spikes mediate the entry of TGEV into host cells through the interaction of a specific domain of the S protein with amino peptidase N (APN), an ectoenzyme abanduntly expressed on the small intestine enterocytes (Delmas, 1990). The S protein therefore mediates APN receptor recognition (Godet et al, 1994). Mutations have been noted in the S encoding gene, which do not affect the replication of the virus in cell cultures but lead to dramatic attenuation of enteropathogenicity. These mutations were noted to occur in the amino acid stretch from residue 145-155 from the mature protein N terminus. Using a recombinant protein containing the amino terminal 330 residues of the S1 sub-unit, Kubo et al (1994) also demonstrated that receptor-binding activity was attributed to these residues. This domain is well conserved in the antigenic cluster I viruses with the exception of PRCV, which turns out to have a tropism for respiratory tissue compared to the rest of the cluster which have a tropism for the digestive tract. It is also possible that other viral or cellular regulatory mechanisms affecting critical steps of viral replication other than virion binding could be located on other genes. These genes could also have an influence on tissue tropisms

Transmembrane Anchor

There is a region of very strong homology in the carboxy-terminus of all the coronaviruses, and this sequence is flanked by the sequence **KWPWYVLW** on the amino terminal side (Figure 7 (a)). The region is strongly hydrophobic and is thought to be the putative membrane anchor (de Groot et al, 1987). The membrane anchor has is more precisely defined in CCV, from residues 1395-1415, has the residues **VWLLIGLVVIFCIPLLLFC**. This region is highly conserved in all coronaviruses in all alignments.

Stem Structure

On the carboxy-terminus side, a cysteine cluster (Figure 7 (b)) flanks the membrane anchor. It has been proposed that at least some of the cysteine residues in this region be involved in the acylation of the S protein (Raaschaert and Laude 1987). Heptad repeat structures have been observed in the S proteins (which have been proposed by (de Groot et al, 1987 and Rasschaert and Laude 1987) to be essential elements in forming the elongated structure of the peplomers. In CCV, there are 2 regions with heptad periodicity (residues 1068-1150 and 1336-1381), which are proposed to be essential elements for the formation of the elongated stem structure (de Groot 1987). The shorter domain adjacent to the transmembrane domain and is a leucine zipper motif (Briton, 1991). The role of this region will only be fully understood when the structure of the S protein has been fully resolved (Wesseling et al, 1994). Delmas and Laude (1990) have led to the proposal that the S protein

is a homotrimer. The S oligomer is possibly stabilized by an interchain coiled-coil elongated structure predicted from the sequence near the C-terminal quarter of the polypeptide chain.

Fusion Peptide

A common feature of viral fusion proteins is the presence of a fusion peptide, which is believed to participate directly in the fusion process. Fusion peptides are typically composed of 16-26 amino acid residues and conserved within virus families. They are relatively hydrophobic and generally show an asymmetric distribution of hydrophobicity when modeled into an alpha helix; they are also rich in alanine and glycine. Fusion peptides can also be internal, and have the same features described above. Furthermore, these internal fusion peptides are bounded by charged residues on both ends and may contain a proline residue in the center. Coronaviruses could possibly have an internal fusion peptide. Luo and Weiss made a mutational analysis of the possible fusion peptides in MHV and examined the effect of the mutations on cell-to-cell fusion, protein processing and cell surface expression. Mutations in these residues **KMIASAFNNALGAIQDGFD** were found to result in failure of cell fusion in MHV and are therefore a likely candidate for a fusion peptide. In the analysis of the sequences in this paper, this region is highly conserved in all the coronaviruses. Residues **HILSLVQNAPYGLYFIH** (also highly conserved in all viruses examined) were noted to play a role in the ability of S proteins to be retained in the ER and eventually expressed on cell surface. Mutations in this region are thought to interfere with these processes (Luo and Weiss, 1998).

Selection of Antigenic Peptides for Oral Viruses

As mentioned earlier, research efforts have recently increased towards determining essential sequences to express in baculoviruses and plants with the ultimate goal of making oral vaccines. In TGEV, four antigenic sites (A, B, C and D), with sites A and D, the major inducers of neutralizing antibodies, being highly preserved in antigenic cluster I. These are therefore candidates for oral vaccines in plants or baculovirus. Site A is antigenically dominant. Some monoclonal antibodies on S detect linear and others conformation dependent epitopes. Site A is both conformation and glycosylation dependant while site D is glycosylation independent (Delmas et al, 1986). The requirement for glycosylation in site A is a critical one but both higher plants and insect cells have been shown to be capable of glycosylation, although they might do this slightly differently than would animal cells. However, site a on its own was shown to be incapable of producing virus neutralizing antibodies in the absence of other immunogenic epitopes, site D in particular. Site D is defined by the residues between amino acid residues 378 to 395 (underlined in Figure 6(b)) in TGEV whereas the residues **MKRSGYGQPIA** are well conserved within the A site. Interestingly, all the viruses that are closely evolutionarily related to TGEV have these residues very well conserved (Figure 5). In view of the S protein's role in binding to host cells, and the fact that sites A and D on this protein induce neutralizing antibodies, Sanchez and co-workers (1990), suggest that these residues must be important for viral replication. This process starts with the binding of virions to host cells.

This conservation of residues in site A and D could potentially increase the utility of an oral vaccine developed using amino acid residues from the immunogenic site. Site D elicits antibodies with four epitope specificities identified by 4 different monoclonal

antibodies. According to Delmas and co-workers (1990) all TGEV strains that they tested maintain one or more of these epitopes, which makes unlikely the appearance of escaping mutants for all the epitopes in one virion.

Conclusions

To summarize, evolutionary relationships among the 12 coronaviruses in the data set used for this study reflect the antigenic clusters that were determined on the basis of serological relationships. These results are not surprising, because S gene protein that was used to determine the evolutionary relationships is the site of immunogenic epitopes and viral binding to host cells. It therefore makes sense that relationships based on this protein should reflect the different antigenic clusters. What was striking about the results is the precision with which the evolutionary relationships match the antigenic clusters. The S gene was therefore a prudent choice of viral protein to use for this purpose, validating the relationships determined on the basis of serological data. Precise correlation of specific sequences and function will require molecular characterization of isolates of the same virus, strains selected for virulence and mutants to locate genomic changes associated with alterations in biological characteristics. We believe that the evolutionary relationships of the coronaviruses we deduced, which reflect serological sub-groupings are the likely phylogeny for the viruses in this study. The bootstrap ratings for the evolutionary tree provide strong support for this.

Aligning sequences helps to identify conserved sequences whose conservation may indicate a functional role. Sequence alignment coupled to protein structure and function could also be a useful tool in the development of oral vaccines. Comparative analyses have not permitted us to identify specify amino acid sequences with functions. However, as more

members and strains of these coronaviruses are sequenced, more solid conclusions about structure function relationships can be made after more in-depth molecular analysis of specific strains/mutants of the same virus with regards structural conformation and tropisms.

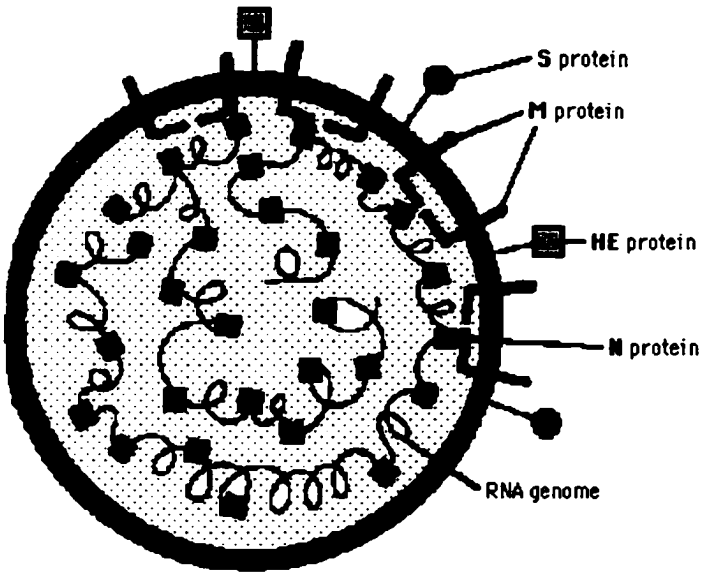

Figure 1. A cartoon representation of a coronavirus particle indicating the structural Proteins (from the web)

N	-	Nucleocapsid protein
M	-	Membrane protein
S	-	Spike protein
HE	-	Hemagglutinating esterase protein (not present in all coronaviruses)

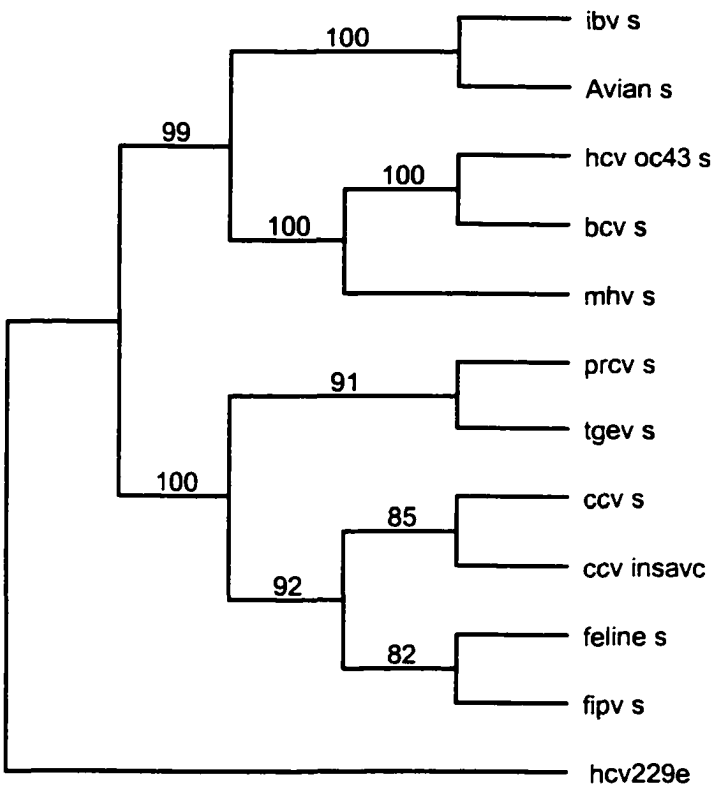

Figure 2. Evolutionary relationships between some coronaviruses. Bootstrap ratings were generated in a heuristic search with 100 replicates and a thousand characters were sampled in each replicate.

Tree Length: 3746
Consistency Index: 0.96
Homoplasy Index: 0.04

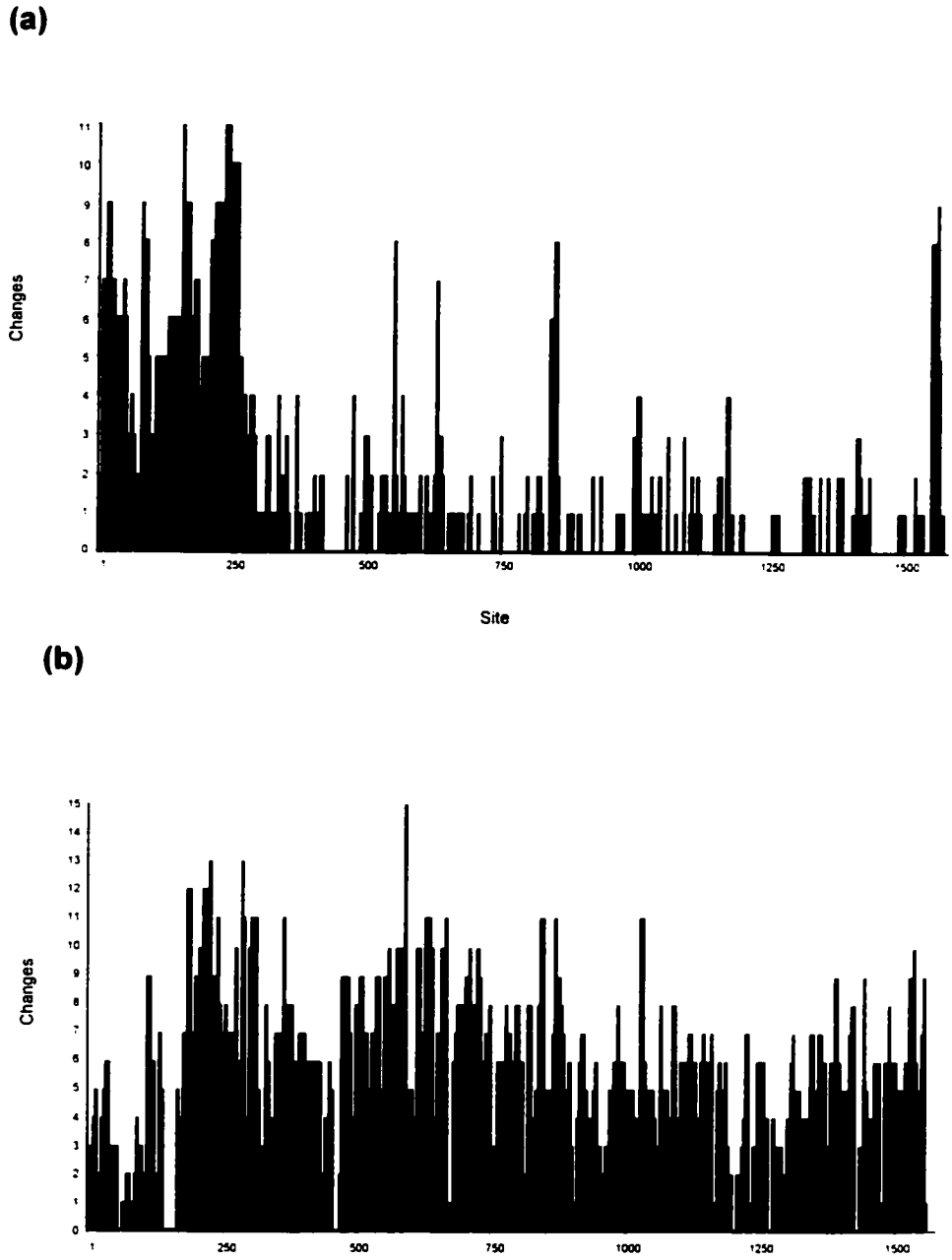

Figure 3. The Amino Acid Variation across the two main clusters of coronaviruses. Variation across

(a) cluster I with a window size of 5 and 1500 residues. There is strong residue conservation in the N-terminus of this cluster

(b) cluster II and III combined with similar residues and window size. The figure shows conservation in the C-terminus.

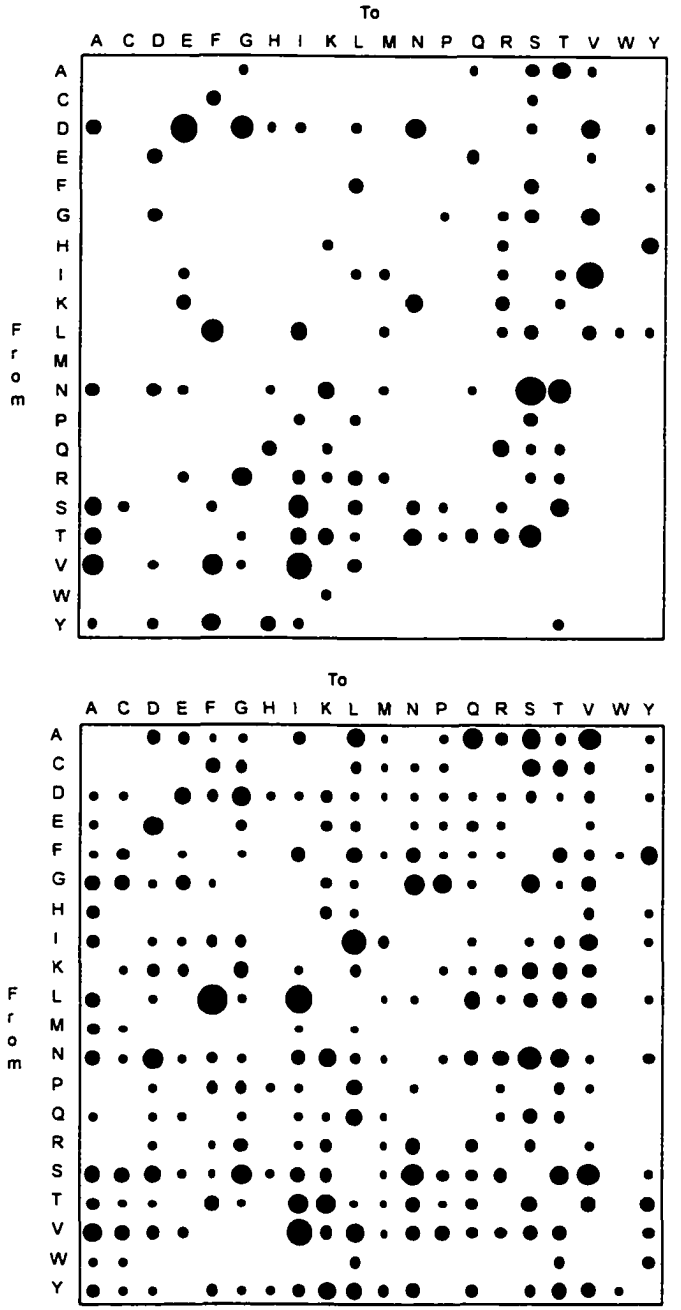

Figure 4. Bubble plots showing unambiguous changes in the two major clusters.
(a) Frequency of unambiguous residue changes for antigenic cluster I.
(b) Frequency of unambiguous residue changes for antigenic cluster II and III combined.

(a) Conserved residues in site A (underlined)

```
ibv_s           VNTTCTLHNFIFHNETGANPNPSGVQNIQTYQTKTAQSGYYNFNFSFLSSFVYKESNFMY
Avian_s         VNTTLTLTNFTFSNESGAPPNTGGVDSFILYQTQTAQSGYYNFNLSFLSSFVYRGSNYMY
prcv_s          SFLTHTIVNITIGLGMKRSGYGQPIASTLSNITLPMQDNNTDVYCVRSDQFSVYVHSTCK
fipv-s          SFFTYTAVNITIDLGMKLSGYGQPIASTLSNITLPMQDNNTDVYCIRSNQFSVYVHSTCK
tgev_s          SFHTHTIVNLTIDLGMKRSGYGQPIASTLSNITLPMQDNNTDVYCIRSDQFSVYVRSTCK
ccv_s           SFYSHTSVNITIDLGMKRSGYGQPIASTLSNITLPMQDNNTDVYCIRSNRFSVYFHSTCK
feline_s        IFFAHTAINITIDLGMKRSGYGQPIASTLSNITLPMQDNNTDVYCIRSNQFSVYVHSICK
ccv-insavc      SFYSHTSVNITIDLGMKRSVTVTI-ASPLSNITLPMQDNNIDVYCIRSNQFSVYVHSTCK
bcv_s           KAPKNFCPCKLDGSLCVGNGPGID-AGYKNSGIGTCPAGTNYLTCHNAAQCDCLCTPDPI
mhv_s           GASYCPCANPSIVSPCTTGKPKFA--NCPTGTT----NRECNVLALGSNLFKCDCTCNPS
hcv_oc43_s      KAPKNFCPC-KLNGSCVGSGPGKN------NGIGTCPAGTNYLTCDNLCTPDPITFTGTY
hcv-229E        VYHKHTFIVLYVDFKPQSGGGKCF-NCYPAGVN----ITLANFNETKGPLCVDTSHFTTK
```

(b) Conserved residues in site D (underlined)

```
ibv_s           MAWSSSQFCTAHCNFSDTTVFVTHCYKHG-GCPLTG---MLQQNLIRVSAMKN--GQLFY
Avian_s         MSWSTASFCTAHCNFTSYIVFVTHCFKSGPSCPLTGLIPSGYIRIAAMKHGSAMPGHLFY
prcv_s          FTTNVQSGKGATVFSLNTTGGVTLEISCYNDTVSDSSFSSYGEIPFGVTNGPRYCYVLYN
fipv-s          FTADVQSGMGATVFSLNTTGGVILEISCYSDTVSESSSYSYGEIPFGITDGPRYCYVLYN
tgev_s          FTTNVQSGKGATVFSLNTTGGVTLEISCYNDTVSDSSFSSYGEIPFGVTDGPRYCYVLYN
ccv_s           FTALVQSGMGATVFSLNTTGGVILEISCYNDTVSESSFYSYGEISFGVTDGPRYCFALYN
feline_s        FTADVQSGMGATVFSLNTTGGVILEISCYNDTVSESSFYSYGEIPFGITDGPRYCYVLYN
ccv-insavc      FTTDVQSGMGATVFSLNTTGGVILEISCYNDTVSESSFYSYGEIPFGVTDGPRYCYVLYN
bcv_s           TGVYELNGYTVQPIADVYRRIPNLPDCNIEAWLNDKS--VPSPLNWERKTFSNC-NFNMS
mhv_s           TGVYDLSGYTVQPVGLVYRRVRNLPDCRIEDWLAAK--TVPSPLNWERKTFQNC-NFNLS
hcv_oc43_s      TGVYELNGYTVQPIADVYRRKPNLPNCNIEAWLNDKS--VPSPLNWERKTFSNC-NFNMS
hcv-229E        LNFEENLRRGTILFKTSYGVVVFYCTNNTLVSGDAHIPFGTVLGNFYCFVNTTIGNETTS
```

Figure 6. Conservation of regions containining critical residues from antigenic sites A and D. Conserved residues are underlined.

```
ibv_s           VP--ILDIDSEIDRIQGVIQGLNDSLIDLEKLSILKTYIKWPWYVWLAIAFATIIFILIL
Avian_s         IP--VLNIIYDIDKIEEVIKGLNDSLIDLETLSILKTYIKWPWYVWLAIFFAIIIFILIL
prcv_s          LEFRSEKLHNTTVELAILIDNINNTLVNLEWLNRIETYVKWPWYVWLLIGLVVIFCIPLL
flpv-s          LEFRSEKLHNTTVELAILIDNINNTLVNLEWLNRIETYVKWPWYVWLLIGLVVVFCIPLL
tgev_s          LQFRSEKLHNTTVELAILIDNINNTLVNLEWLNRIETYVKWPWYVWLLIGLVVIFCIPLL
ccv_s           LEFRSEKLHNTTVELAILIDNINNTLVNLEWLNRIETYVKWPWYVWLLIGLVVIFCIPIL
feline_s        LEFRSEKLHNTTVELAILIDNINNTLVNLEWLNRIETYVKWPWYVWLLIGLVVIFCIPLL
ccv-insavc      LEFRSEKLHNTTVELAILIDNINNTLVNLEWLNRIETYVKWPWYVWLLIGLVVIFCIPIL
bcv_s           FL--DLQDEMNRLQEAIKVLNQSY--INLKDIGTYEYYVKWPWYVWLLIGFAGVAMLVLL
mhv_s           VTFLDLSDEMNRIQEAIKKLNESY--INLKEIGTYEMYVKWPWYVWLLIGLAGVAVLCVLL
hcv_oc43_s      FL--DLQVEMNRLQEAIKVLNQSY--INLKDIGTYEYYVKWPWYVWLLICLAGVAMLVLL
hcv-229E        LENKSAELNYTVQKLQTLIDNINSTLVDLKWLNRVETYIKWPWWVWLCISVVLIFVVSML
                                           *       * **** *** *          *
```

*Indicates perfectly conserved residues in all the sequences in the database.

Figure 7 (a). Highly conserved regions in the C -terminus domain, which is strongly hydrophobic and is thought to be the putative membrane anchor.

```
ibv_s           GWVFFMTGCCGCCCGCFGIMPLMSKCGKKSSYYTTFDNDVVTEQYRPKKSVSKVPPPSYF
Avian_s         GWVLLMTGCCGCCCGCFGIIPLMSKCGKKSSYYTTFDNDVVTEQYRPYYSVFTPQYLSCL
prcv_s          LFCCCSTGCCGCI-GCLGSCCHSIFSRRQFENYEPIEKVHVH------------------
flpv-s          LFCCFSTGCCGCI-GCLGSCCHSICSRRQFENYEPIEKVHVHISGCINSLRTPLISHYPC
tgev_s          LFCCCSTGCCGCI-GCLGSCCHSICSRRQFENYELIEKVHVHINVNFIKLSSHYIVPLHL
ccv_s           LFCCCSTGCCGCI-GCLGSCCHSICSRRQFESYEPIEKVHVH------------------
feline_s        LFCCCSTGCCGCI-GCLGSCCHSMCSRRQFENYEPIEKVHVHIIRQLFLLENTSELNL--
ccv-insavc      LFCCCSTGCCGCI-GCLGSCCHSICSRGQFESYEPIEKVHVH------------------
bcv_s           FFICCCTGCGTSCFKICGGCCDDYTGHQELVIKTSHDDVRLFI----------------
mhv_s           FFICCCTGCGSCCFKKCGNCCDEYGGHQDSIVIHNISSHED------------------
hcv_oc43_s      FFICCCTGCGTSCFKKCGGCCDDYTGYQELVIKTSHDDVRLFIALISCIFLQSSICSSGH
hcv-229E        LLCCCSTGCCGFF-SCFASSIRGCCESTKLPYYDVEKIHIQWLVCSHCNLCLL-------
                      ***
```

Figure 7(b). Cysteine rich domain thought to be important in the formation of heptads repeats required for the stem structure of the S protein.

Table 1. Antigenic Clusters of coronaviruses (from Holmes and Lai, 1996)

Antigenic Group	Virus	Host	Respiratory Infection	Enteric infection	Hepatitis	Neurologic Infection	Other
I	HCV-229E	Human	X				
	TGEV	Pig	X	X			X
	PRCV	Pig					
	CCV	Dog		X			
	FECV	Cat		X			
	FIPV	Cat	X	X	X	X	X
	RbCV	Rabbit					X
II	HCV-OC43	Human	X	?		X	
	MHV	Mouse	X	X	X	X	
	SDAV	Rat				X	
	HEV	Pig	X	X			
	BCV	Cow		X			
	RbEVC	Rabbit		X			
	TCV	Turkey	X	X			
III	IBV	Chicken	X		X		X

Abbreviations

TGEV - transmissible gastroenteritis virus
PRCV - porcine respiratory coronavirus
MHV - murine hepatitis virus
FIPV - feline infectious peritonitis virus
FECV - feline enteric coronavirus
HCV - human respiratory coronavirus (HCV strains 229E and OC43).
BCV - bovine coronavirus
CCV - canine coronavirus (CCV strains Insavc and K378).
IBV - infectious bronchitis virus (avian)
RbCV - Rabbit coronavirus
RbECV - Rabbit Enteric Coronavirus
HEV - Porcine hemmaglutinating encephalomyelitis virus

References

Ballesteros, M.L., Sanchez, C.M. and Enjuanes, L. (1997). Two amino acid changes at the N-terminus of transmissible gastroenteritis coronavirus spike protein result in the loss of enteric tropism. Virology 227 (2): 378-388.

Baric R.S., Fu, K., Schaad, M.C., Stohlman, S.A. (1990). Establishing a genetic recombination map for murine coronavirus strain A59 complementation groups. Virology 177: 646-656.

Buchmeier, M.J., Lewicki, H.A., Talbot, P.J. and Knobler, R.L. (1984). Murine hepatitis virus-4 (strain JHM) induced neurologic disease is modulated in vivo by monoclonal antibody. Virology 132: 261-270.

Callebaut, P., Enjuanes, L. and Pensaert, M., (1996). An adenovirus recombinant expressing the spike glycoprotein of porcine respiratory coronavirus is immunogenic in swine. Journal of General Virology, 77: 309-313.

Correa, I., Jimenez, G., Sune, C., Bullido, M.J and Enjuanes, L. (1988). Antigenic structure of the E2 glycoprotein from transmissible gastroenteritis coronavirus. Virus Research 10: 77-94.

Correa, I., Gebauer, F., Bullido, Sune, C., M.J, Baay, M.F.D., Zwaagstra, K.A., Posthumus, W.P.A., Lenstra, J.A. and Enjuanes, L. (1990). Localization of antigenic sites of the E2 glycoprotein of transmissible gastroenteritis coronavirus. Journal of General Virology, 71: 271-279.

De Groot, R.J., Luytjes, W., Horzinek, M.C., van der zeijst, B.A.M., Spaan, W. J., and Lenstra, J.L. (1987). Evidence for a coiled coil structure in the spike protein of coronaviruses. Journal of Molecular Biology 196: 963-966.

Delmas, B., Rasschaert, D., Godet, M., Gelfi, J. and Laude, H. (1990). Four major antigenic sites of the coronavirus transmissible gastroenteritis virus are located on the amino terminal half of the spike glycoprotein S. Journal of General Virology 71: 1313-1323.

Delmas, B., Gelfi, M. and Laude, H.(1986). Antigenic structure of transmissible gastroenteritis virus. II. Domains in the peplomer glycoprotein. Journal of General Virology 67:1405-1418.

Enjuanes, L., and Van der Zeijst, B.A.M., (1995). Molecular basis of transmissible gastroenteritis coronavirus (TGEV) epidemiology, p. 337-376. In S.G. Siddell (ed.). Coronaviruses. Plenum Press, New York.

Felsenstein, J., (1985). Confidence limits on phylogenies: An approach using the bootstrap. Evolution 39: 783-791.

Gerna, G., Passarani, N., Battaglia,M., and E.G. Rondanelli, (1985). Human enteric coronaviruses: antigenic relatedness to human coronavirus OC43 and possible etiologic role in viral gastroenteritis. J. Infect. Dis. 151; 796-803.

Holmes, K.V and Lai, M.M.C., (1996). Coronaviridae: The viruses and their replication. In Fields, B.N., Knipe, D.M., Howley, P.M. et al (eds). Fields Virology, Third Edition. Lippincott-Raven Publishers, Philadelphia.

Jacobs, L., De Groot, R.J., van der Zeijst, B.A.M., Horzinek, M.C., and W. Spaan, (1987). The nucleotide sequence of the peplomer gene of porcine transmissible gastroenteritis virus (TGEV): comparison with the sequence of the peplomer protein of feline infectious peritonitis virus (FIPV). Virological Research 8: 363-371.

Jimenez, G., Correa, I., Melgosa, M.P., and Enjuanes, L. (1986). Critical Epitopes in porcine transmissible gastroenteritis virus neutralization. Journal of Virology 60: 131-139.

McIntosh, K. (1974). Coronaviruses: A comparative review. Current Topics in Microbiology and Immunology 63: 85-129.

Paul, P.S., Zhu, X.L., and Vaughan, E., 1988. Current Strategies for the Development of Efficacious Vaccines for Transmissible Gastroenteritis in Swine. In: Proceedings of the 92nd Annual Meeting of the US Animal Health Association, Little Rock, Arkansas, pp. 429 - 443.

Paul, P.S., Zhu, X.L., and Vaughan, E., (1994). Significance of Porcine Respiratory Coronavirus Infection. Continuing education for the practical Veterinarian 16(9): 1223-1234.

Pederson, N.C., Everman, J.F., McKeirnan, and Ott, R.L., (1984). Pathogenicity studies of feline coronavirus isolates 79-1146 and 79-1683. American Journal of Veterinary Research 45: 2580-2585.

Raabe, T., Schelle-Prinz, B. and Siddell, S.G. (1990). Nucleotide sequence of the gene encoding the spike glycoprotein of human coronavirus HCV-229E. Journal of General Virology 71: 1065-1073.

Rasschaert, D and Laude, H. (1987). The predicted primary structure of the peplomer protein E2 of the porcine coronavirus transmissible gastroenteritis virus. Journal of General Virology 68: 1883-1890.

Sanchez, C.M., Jiminez, G., Laviada, M.D., Correa, I., Sune, C., Bullido, M.J., Gabauer, F., Smerdou, C., Callebaut, P., Escribano, J.M. and Enjuanes, L. (1990). Antigenic

homology among coronaviruses related to transmissible gastroenteritis virus. Virology 174: 410-417.

Sanchez, C.M., Jiminez, G., Correa, I., Sune, C., Mendez, A., Dopazo, J. and Enjuanes, L. (1992). Genetic evolutionand tropism of Transmissible Gastroenteritis Coronaviruses. Virology 190: 92-105.

Shoup, D.I, Jackwood, D.J. and Saif, L.J., (1997). Active and passive immune responses to transmissible gastroenteritis virus (TGEV) in swine inoculated with recombinant baculovirus-expressed TGEV spike glycoprotein viruses.

Schmidt, O.W., and Kenny, G.E. (1982). Polypeptides and functions of antigens from human coronaviruses 229E and OC43. Infect.immun. 35:515-522.

Spaan, W., Cavanah, D., and Horzinek, M.C. (1988). Coronaviruses: Structure and Genome expression. Journal of General Virology 69: 2939-2952.

Tacket, C.O., Mason, H.S., Losonsky, G, Clements, J.D., Levine, M.M. and Arntzen, C.J., (1998). Immunogenicity in humans of a recombinant bacterial antigen delivered in transgenic potato. Nature Medicine 4:607-609.

Torres, J.M., Sanchez, C.M., Sune, C., Smerdou, C., Prevec, L., Graham, F.,and Enjuanes, L. (1995). Virology 213:503-516.

Torres, J.M., Covadonga, A., Ortega, A., Mittal, S., Graham,F., and Enjuanes, L. (1996). Tropisms of Human Adenovirus Type 5-based vectors in swine and their ability to protect against transmissible gastroenteritis coronavirus. Journal of Virology 3770-3780.

Tuboly, T., Nagy, E., Dennis, J.R. and Derbyshire, (1994). Immunogenicity of the S protein of transmissible gastroenteritis virus expressed in baculovirus. Archives of Virology 137: 55-67.

Welter, L.M., Mason, H.S., Lu, W., Lam,D.M.K., Welter, M.W. (1996). Effective immunizations of piglets with transgenic potato plants expressing a truncated TGEV S protein. In: Vaccines: New Technologies and Applications. (Cambridge Health Institute).

Wesseling, J.G., Vennema, H., Godeke, G., Horzinek, M. and Rottier, P.J.M. (1994). Nucleotide sequence and expression of the spike (S) gene of canine coronavirus and comparison with the S proteins of feline and porcine coronaviruses. Journal of General Virology 75:1789-1794.

CHAPTER 7

General conclusions

This work examines the possibility of using transgenic maize as a source of functional antigens that could potentially be used as oral vaccines for livestock and humans. The heat labile toxin (LT) from *E. coli* and its subunits (LT-A, LT-B) and the N terminal domain of the TGEV S protein were used as model antigens. Work presented in this dissertation examined some basic aspects of expression of a foreign gene in transgenic maize, such as promoters directing gene expression, characterization of transgenic plants and evaluation of the transgene expression in several generations of transgenic plants from select transgenic events. Biochemical, biophysical and immunogenic properties of the maize synthesized antigens were determined.

Chapter 2 establishes the possibility of expressing a synthetic LT-B gene in maize. Two different promoters, the constitutive CaMV 35S promoter and the seed specific 27 kDa gamma zein promoter were used to express the LT-B gene in maize. Evaluation of levels of LT-B expression showed that the seed specific gamma zein promoter was more effective than the CaMV 35S promoter in achieving high-level expression in maize kernels. The combination of the SEKDEL signal motif and the seed specific promoter resulted in enhanced LT-B gene expression in the first generation of transgenic plants. The stability of LT-B gene expression in several generations was studied in selected transgenic events. The general trend observed was that the LT-B expression increased several fold from the R_1 putative to the R_3 generation. The explanation put forward for this observation is that

subsequent generations are free from the stress of in *vitro* culture and that at each stage, only the expressing individuals were planted. Significant variation was observed between transgenic events. This was attributed to differences in transgene copy number and site of transgene integration within the genome of transgenic plants. Significant differences were also observed between individual plants within the same event. This is a phenomenon that is not easily explained, but has been observed by other groups working with transgenic maize. The results of this section demonstrated the ability of maize to synthesize LT-B with the same biophysical and biochemical features as the native bacterial LT-B.

The role of signal peptides in determining the subcellular localization of LT-B in transgenic maize kernels is presented in Chapter 3. Subcellular localization is important for the proper assembly, accumulation and functionality of a protein in any expression system. The study determined the subcellular location of LT-B protein fused to either the bacterial or zein signal peptide. Immunogold labeling/electronmicroscopy and western analysis revealed that LT-B in transgenic maize was localized in starch granules. The results from this work present strong evidence that the bacterial signal peptide targets the LT-B protein to the starch granules in maize endosperm. To our knowledge, this is the first report of a bacterial signal peptide targeting a protein to the starch granules of a high plant. Further experimentation is required to make a conclusive determination that the bacterial signal peptide directs LT-B to maize endosperm starch. Encapsulation of LT-B has important implications for the production of other antigens, biopharmaceuticals or other industrial proteins in starch granules. The observation also potentially provides another tool for studying protein translocation.

Immunogenicity of maize generated LT-B was determined through a mice feeding

experiment. This work, presented in Chapter 4, showed that maize synthesized LT-B was similar to the native LT-B and could induce strong mucosal and serum antibody when orally administered in mice. The transgenic maize synthesized LT-B induced a much stronger immune response than an equivalent amount of bacterial LT-B spiked into transgenic maize. We speculate that the starch encapsulation of LT-B in transgenic maize could have protected the LT-B from the acidity and degradative enzymes of the stomach, and that its slow release from starch could have prolonged exposure to the gut associated immune system. This resulted in the significantly higher immune response observed.

Having established the capacity of maize to synthesize properly folded immunogenic protein, the next phase of research focused on the design of a recombinant subunit vaccine against swine transmissible gastroenteritis. This work is described in Chapter 5. A mutant, non-toxic LT holotoxin was expressed in transgenic maize callus, and it was demonstrated that the holotoxin could be assembled in maize. The next step was to replace the A1 subunit of the mutant holotoxin and replace this with an immunogenic fragment from the S protein of the porcine respiratory coronavirus bearing TGE virus-neutralizing antibodies. The objective was to make a recombinant holotoxin utilizing the immunogenic, adjuvant and delivery capacity of LT-B. The result of this work was co-expression of the antigen/recombinant A subunit and the LT-B subunit in maize callus. No TGE neutralizing antibodies were observed. It was concluded that the recovery of transgenic events with low TGEV peptides could be the reason for this observation.

Chapter 6 presents an analysis of the evolutionary relationships of coronaviruses based on their spike protein, using TGEV as the reference protein. Results of this analysis show an evolutionary tree similar to that observed when serological methods are used to

establish this relationship. The analysis also shows that the critical epitopes required for virus neutralization are strongly conserved in coronaviruses in the same antigenic structure. Although sequence alignment is not commonly used for elucidation of structure function relationships in viruses, it is becoming increasingly common in fields such as bioinformatics. Such analysis could have important implications in elucidation of protein structure and contribute to the determination of residues required for determination of such important aspects as tissue tropisms.

Appendix I describes regulatory aspects relating to the release of transgenic plants into the environment. Formal proceedings were followed to obtain a permit for releasing transgenic plants carrying the LT-B gene into the environment. These plants were grown in the field in the summer of 2001 in accordance to the regulations stipulated by USDA-APHIS.

The work presented in this dissertation demonstrates that maize can be used as a production and delivery vehicle for the production of functional antigens. Targeting antigen expression to kernels offers an opportunity to produce the antigens in an industrially convenient tissue for processing. Regulatory sequences can be used to enhance expression of the antigens and the use of standard agronomic and plant breeding techniques can result in the production of stable transgenic lines with the desired levels of novel protein. Subcellular localization of antigens can be manipulated to target organelles in which the antigens are protected from the stomach environment and to enhance exposure to the immune system in the gut. This study also made available transgenic maize germplasm expressing LT-B protein to be used for co-administration with other antigens or conventional vaccines. The germplasm can also be used in transformation and breeding programs to achieve coexpression with other antigens to be used as vaccines. Expression and assembly of

LTK63, a more complex molecule than LT-B, illustrates that maize expressed antigens are capable of higher order folding. Because of the pivotal role of maize as a major ingredient in livestock feed, expression of antigens in maize kernels can have a major impact in the administration of vaccines to commercial herds. In other countries, maize is a staple of many diets and therefore there is great potential to impact development and delivery of human vaccines.

APPENDIX

FIELD RELEASE OF LT-B EXPRESSING CORN

1 Introduction

Transgenic plants have become attractive systems for the production of vaccines and therapeutic proteins for humans and livestock. However, there are potential risks associated with this technology that have led to the development of guidelines to regulate the release of the genetically engineered crops into the environment. The general concern is the possibility of transfer of the transgenes from the engineered crops into the weedy relatives, among other concerns. For transgenic plants carrying genes that encode biologics (antigens for use as vaccines, antibodies) or biopharmaceuticals, the concern has been that of the transfer of the genes into seed and commercial crops.

Indeed, the possibility of gene transfer from transgenic crops to weedy relatives and other crops have been demonstrated (Mikkelsen et al, 1996, Chevre et al, 1997). The potential risks can be reduced using a variety of technologies. These technologies will not be addressed in this dissertation, but there are several possible options including the use of promoters that govern spatial and temporal expression of the transgenes and even strategically restricting the period of transgene expression.

While experimentation is ongoing to provide data to separate real and perceived risks regarding environmental release of genetically modified plants, compliance with the regulations that are currently in place is a major tool in minimize these risks. During my

PhD training I interacted with the Animal and Plant Health Inspection Service (APHIS), the US government department overseeing the implementation of safety regulations to minimize the environmental risk posed by the release of transgenic organisms into the environment. Field planting/testing of transgenic crops usually requires a Notification to APHIS. However, in the case of transgenic plants containing biopharmaceuticals and other biologics, a release permit is required. In March of 2001, I initiated proceedings to obtain a permit to field test the LT-B expressing corn under the close supervision of my major Professor, Dr Kan Wang. The following is the information required to support the application.

2 Procedure

The procedure was initiated by submitting to APHIS a form designated Form 2000, which is available from the APHIS website. This form is accompanied by enclosures, providing all the information that is required before the release permit is issued. I researched for and provided this information with the help of Dr Kan Wang. The following section provides this information.

2.1 Enclosures

a. ***Names Addresses and Telephone Numbers of Responsible Persons***

b. ***A description of the anticipated or actual expression of the altered genetic material in the regulated article and how that expression differs from the expression in the non-modified parental organism.***

The regulated article in this request is transgenic corn carrying a subunit gene from the E. coli heat labile enterotoxin (LT-B corn). This work is supported by USDA-NRI (Grant #99-35504-7799). The purpose of this project is to develop a corn-based oral vaccine delivery system for livestock.

The expression of the LT-B gene under the maize gamma-zein promoter will accumulate LT-B subunit protein in endosperm of corn seed. Non-transgenic corn does not contain this protein naturally. No LT-B protein has been detected in any other part of corn plant.

No phenotypical difference (in terms of height, color or other morphological changes) of LT-B corn was and should be observed in whole plant or seed.

c. A detailed description of the molecular biology of the system (e. g., donor-recipient-vector), which is or will be used to produced the regulated article.

The Gene

LT-B is part of the LT toxin produced by enterotoxigenic strains of *E. coli*, a bacterium responsible for producing traveler's diarrhea, mostly in developing countries. The LT toxin is an 84-kilodalton polymeric protein composed of 2 major non-covalently associated immunologically distinct regions or domains designated (LT-A and LT-B). The A region (27 kDa) is responsible for the diverse biological effects of the toxin. LT-B is a 55kDa region and is responsible for binding of the toxin to the host cell receptor, GM1 (galactosyl-N-acetylgalactosaminyl-sialyl galactosyl glucosyl ceramide), which is found on the surface of all eukaryotic cells (Clements and Finkelstein, 1979).

Although the LT-B protein is a constituent of the E. coli toxin holoenzyme, the LT-B

protein alone has no toxic activity and cannot cause disease (Spangler, 1992). However, it can stimulate immune system of mammal so it can be used as a vaccine against the disease. It was reported that transgenic potato carrying LT-B protein could be used as a vaccine for human for prevention of travelers' disease (Mason et al, Nature Medicine, 1998).

The construct

The constructs introduced to corn plants are shown in Appendix 1 and 2. The codon-optimized LT-B gene (Mason et al, Vaccine, 1998) was cloned behind the maize gamma zein promoter and terminated by the soybean vegetative storage terminator. This construct was named as pRC4. The vector for this construct was the commonly used vector pUC19.

Transformation

The transgenic corn plants were produced using microprojectile bombardment (Frame et al, 2000). The construct pRC4 was co-transformed with pBAR184, a construct carrying the selectable marker (see below for detail).

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Selectable genetic marker genes

The genetic constructs include the well-characterized genetic marker gene *bar*, which encodes the enzyme phosphinothricin acetyl transferase and confers resistance to the herbicides Basta or its active ingredient Bialophos. This marker gene is used to facilitate selection of transformed plant tissues in the laboratory. The bar gene is derived from the bacterium *Streptomyces hygroscopicus* (Murakami et al, 1986).

Non-coding DNA regulatory sequences

Some of the DNA regulatory sequences introduced into the transgenic corn plants were derived from the corn, the 27kD gamma zein promoter (Larkins et al, 1985) and the VSP terminator is part of the regulatory sequence of the soybean vegetative storage proteins (Mason et al, 1993). The bar gene is regulated by the maize ubiquitin promoter and the nopaline synthase terminator from *Agrobacterium tumefaciens* nopaline synthase gene in the vector pUC8 (Messing et al, 1985)). None of the regulatory sequences introduced into the transgenic maize lines can cause plant disease by themselves or in conjunction with the genes, which they regulate.

Gene Copy numbers

Three independent transgenic events with transgene copy number ranging from 2-5 as characterized by Southern blot analysis in our laboratory, and the introduced genes should remain stably integrated in the corn genome.

d. Country and locality where the donor organism, recipient organism, and vector or vector agent were collected, developed and produced.

The constructs pRC4 and pBAR184 were developed in the Plant Transformation Facility of the Department of Agronomy at Iowa State University, Ames, Iowa 50011-1010

e. A detailed description of the purpose of the introduction of the regulated article including a detailed description of the proposed experimental and/or production design.

The field release of the regulated article will be for the purpose of bulking of seed for further molecular and biological studies for this project. We are interested in developing a corn-based edible vaccine system for livestock. The future study aims at optimizing production and accumulation of vaccine in transgenic corn using LT-B protein as a model system. We will analyze the biochemical properties including antigenecity and immunogenicity of the transgenic protein as well as the glycosilation system in corn plant. We also want to look at the stability of expression of LT-B in field conditions

Using transgenic plants to produce industrial or therapeutic biomolecules is one of fastest developing areas in agricultural biotechnology. The advantages of expression and production of these molecules in plants include 1) safe; 2) less costly; 3) can be scaled up; and 4) product maybe stable for long time.

There are no immediate plans to commercialize these plants.

Similar research using other transgenic plant was reported before. One example is transgenic alfalfa plants carrying cholera toxin B subunit (CT-B). The cholera toxin is produced by the bacterium *Vibrio Cholerae*, the causative agent of cholera disease. The CT-B component that was been engineered into alfalfa plants and field-tested by the Noble Foundation under APHIS permit #92-185-01R. APHIS issued a finding of no significant environmental impact when an environmental impact assessment was carried out for CTB.

f. ***A detailed description of the processes, procedures, and safeguards which have been used or will be used in the country of origin and in the United States to prevent contamination, release, and dissemination in the production of the donor organism; recipient organism; vector or vector agent; constituent of each regulated article which is a product; and regulated article.***

The field release is scheduled begin in May 15 of 2001, on a site in Story County, IA. The process should be completed by October 31, 2001. The proposed field test is a controlled release of the regulated article into the environment. The procedures for containment of the plant material and the procedures for termination of the field test, as described (Enclosure #h), should be sufficient to ensure that none of the modified plants persist in the environment. In corn, genes can escape by the wind-borne pollen or the persistence of seed in the environment. The measures described below (Enclosures #h and #I) will minimize dissemination of pollen to receptive, sexually compatible and persistence of the plant material after the conclusion of the test.

g. ***A detailed description of the intended destination (including final and all intermediate destinations), uses, and/or distribution of the regulated article.***

The regulated article is now stored in a locked seed storage room in the Agronomy Hall at Iowa State University.

For field release, the seed of regulated article will be germinated in small pots in the greenhouse of Agronomy Department. We will then transplant 200-300 corn plants that

carrying the LT-B gene to the field.

The field release will take place on agricultural land at the Iowa State University transgenic nursery, Story County, IOWA. An electrical fence to prevent animals or unauthorized personnel from entering will enclose the site. Frequent site monitoring of the field trial and agronomic management practices that create a non-propagative environment are expected to provide the necessary degree of both biological and physical containment. These factors are described below (Enclosure #h).

h. A detailed description of the proposed procedures, processes, and safeguards, which will be used to prevent escape and all dissemination of the regulated article at each of the intended destination.

Plot design

The total area required for this project will not exceed one-fifth of an acre in which 500 transgenic plants will be in rows.

Breeding procedure

The transgenic plants will be either selfed or crossed to their sibling plants, so both the silk and tassels will be bagged individually during the crossing season. This plot will be isolated by a minimum of 400 meters from any other corn plants to prevent the unwanted introduction of transgenic material into non-target plants. An isolation distance of 400 meters is double the isolation distance prescribed for the production of certified corn seed in the United States.

Agricultural practices

Agricultural practices consistent with growing healthy corn plants will be used. The plot will be kept reasonably weed free by mechanical or hand weeding. If necessary, pesticides such as insecticides and/or fungicides will be used to control pests such as corn rootworm, corn leaf aphids, European corn borer, earworms, and various leaf diseases that would reduce the health of the plant and subsequent grain yield. Any pesticides used will be applied by personnel trained in their use and application. The plot will be inspected weekly at first, and then daily during the pollination period.

Field Observation and Monitoring

During the field tests, authorized personnel will inspect the test site at least once a day and will report on seedling emergence, plant vigor, physiological status, reproductive development, disease incidence, insect infestations and bird and small mammal damage.

Security of the field test plot

An electrical fence will be used for isolation of the plot from unexpected or unauthorized entering of animal or human.

i. ***A detailed description of the proposed method of final disposition of the regulated article.***

Any pollinated ears from this plot will be hand-harvested at maturity. The ears will be placed in doubled nylon harvest bags both of which would be independently capable of

preventing seed loss from the ears. After harvest, any remaining plant material will be mowed and incorporated into the soil using a disc or chisel plow.

Following harvest, the test site (including the 10-meter surrounding border zone) will be monitored for any volunteer plants. The plot area will be flagged after harvest and monitored for volunteers throughout the following growing season (2002). In order to facilitate easy identification of any volunteer transgenic plants from the 2001 test, the test plot and surrounding 10-meter border will not be planted with corn in the 2002 season. Any volunteer corn plants will be destroyed before they produce pollen.

Any seed being carried to and from the two fields will be double-bagged to ensure no seed spillage. All seeds harvested from field will be transported and stored in a locked seed storage room in Agronomy Hall of Iowa State University.

All transgenic seeds will be processed to evaluate level of LT-B expression, and the biochemical and immunological properties of the corn derived LT-B will be compared to that of bacteria derived LT. Seeds will be destroyed and rendered nonviable during the process of extraction of LT-B. Any transgenic seeds not used for extraction will be stored securely or devitalized before disposal.

3 Field planting and harvesting

Transgenic maize expressing LT-B subunit protein was grown from May 15 to October 31, 2001 at the Transgenic Nursery at Iowa State University in Washington Township , Story County, IA. Two hundreds transgenic corn seeds were planted in May 29, 2001. Because of the adverse weather at the time of planting, only 135 actually germinated. Sixty five more seeds were planted on June 18th, 54 of these actually germinated. A total of 189 transgenic plant were raised. The plot was isolated by a minimum of 400 meters from any other non-

transgenic corn plants to prevent the unwanted introduction of transgenic material into non-target plants. During the entire period of field release, a two-strand electrical fence that encloses the site was used to prevent animals or unauthorized personnel from entering. Transgenic corn plants were harvested on October 16, 2001 according to the regulations stipulated by APHIS. The Field Manager will continue to monitor the plot for any volunteer plants for the next growing seasons.

4 References

Clements, J.D., and Finkelstein, R.A. (1979). Isolation and characterization of homogenous heat labile enterotoxins with high specific activity from *Escherichia coli* cultures. Infection and immunity: **24**, 760-769.

Chevre et al, 1997. Gene flow from transgenic crops. Nature **389**: 924

Frame, B., Zhang, H., Cocciolone, S., Sidorenko, L., Dietrich, C., Pegg,E., Zhen, S., Schnable, P., Wang, K. (2000) Production of transgenic maize from bombarded type II callus: Effect of gold particle size and callus morphology on transformation efficiency. In Vitro Cell. & Devel. Biol. -Plant. **36**:1-9.

Larkins, B.A., Lending, C.R., Wallace, J.D., Galili., G., Kawata, E.E., Geetha, K.B., Kriz, A.L., Martin, D and Bracker, E.B., (1989). Zein gene expression during maize endosperm development. The Molecular Basis of Plant Development, pp 109-120.

Mason, H.S., Haq, T.A., Clements, J.D., and Arntzen, C.J. (1998). Edible vaccine protects mice against Escherichia coli heat labile enterotoxin (LT): potatoes expressing a synthetic LT-B gene. Vaccine:**16**(3), 1336-1343.

Mason, H.S., DeWald, D.B., and Mullet, J.E., (1993). Identification of a Methyl Jasmonate -Responsive Domain in the Soybean vspB Promoter. The Plant Cell: **5**, 241-251.

Mikkelsen et al (1996) The risk of transgene spread. Nature **370**:31.

Mukarami T, T., Anzai, H., Imai,S., Satoh, A., Nagaoka, K., and Thompson, C.J., (1986). The bialaphos biosynthetic genes of Streptomyces hygroscopicus: molecular cloning and characterization of the gene cluster. Molecular and General Genetics: **205**, 42-50.

Spangler, B.D., (1992). Structure and function of Cholera Toxin and the related Escherichia coli heat labile enterotoxin (LT). Microbiological Reviews: **56**(4), 622-647.

Tacket, C.O., Mason, H.S., Losonsky, G., Clements, J.D., Levine M.M. and Arntzen, C.J. (1998). Immunogenicity in humans of a recombinant bacterial antigen delivered in a transgenic potato. Nature Medicine: **4**(5), 607-609.

Yanisch-Perron, C., Vieira, J. and Messing, J., (1985). Improved M13 phage cloning vectors and host strains: Nucleotide sequence of the M13mp18 and pUC19 vectors. Gene **33**, 103-119.